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SAID SELECTED EPITOPES

(57) Abstract

    The present invention relates to the field of molecular biology and immunology. In particular it relates to vaccines and methods for
providing vaccines which elicit immune responses when administered to a mammal, in particular a human. The preferred elicited immune
response is a T cell response, elicited by peptide T cell epitopes. These vaccines find their application in many fields ranging from cancer
treatments to treatments of prophylaxis of infectious diseases such as Aids. The present invention provides novel methods for selecting the
peptide sequences from an intact antigen which will lead to a proper (T cell) immune response upon administration in a suitable vehicle.
The epitopes and vaccines are, of course, also part of the present invention.

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Title: Methods for selecting and producing T cell peptide epitopes and vaccines incorporating said selected epitopes.

§1 Field of the invention

The present invention relates to the field of molecular biology and immunology. In particular it relates to vaccines and methods for providing vaccines which elicit immune

5   responses when administered to a mammal, in particular a human. The preferred elicited immune response is a T cell response, elicited by peptide T cell epitopes. These vaccines find their application in many fields ranging from cancer treatments to treatments or prophylaxis of infectious

10   diseases such as Aids. The present invention provides novel methods for selecting the peptide sequences from an intact antigen which will lead to a proper (T cell) immune response upon administration in a suitable vehicle. The epitopes and vaccines are, of course, also part of the present invention.

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§2 Background of the invention

Virtually all currently available vaccines are not rationally designed in the sense of detailed knowledge of minimal essential epitopes and the rules of antigen

20   processing and presentation. Rather, the available vaccines are based on empirical knowledge of protection. A major objective of the present invention is to develop a new generation of more rationally designed vaccines which are effective, safe, easy to manufacture and standardise, stable,

25   inexpensive and associated with long lasting protection. This objective is achieved by employing our knowledge on the biochemistry of antigen processing and presentation in general, and in dendritic cells in particular, in the selection of peptide epitopes. Subsequently, selected peptide

30   epitopes are incorporated into various types of vaccines and tested for efficacy in for instance HLA-transgenic mouse models.

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The selection of peptide epitopes for a given combination of antigen and HLA class I molecule according to the invention may be divided in the following subsequent steps:

5   1.   Computer prediction of peptides within the primary sequence of the antigen that are most likely to bind to the HLA class I molecule concerned, by comparison with the relevant motif for MHC class I binding (1).

2.   Measurement of the actual binding of the selected

10   peptides to the MHC molecule concerned using assays that determine HLA-peptide binding and stability of the HLA-peptide complex (2, 3) (Examples 1 and 2 herein).

3.   Screening of the peptides (selected by steps 1 & 2) and their flanking sequences (in the context of the intact

15   antigen) for compliance with the rules for proteasome cleavage of natural protein sequences (4).

4.   Screening of the peptides (selected by steps 1 & 2) and their flanking sequences (in the context of the intact antigen) for compliance with the rules for effective peptide

20   transport and loading into HLA class I molecules (5).

Selected peptide epitopes (see steps 1-4) are incorporated into the following prototype vaccines, the efficacy of which is compared in the appropriate HLA

25   transgenic mouse model:

i.   Mixture of synthetic peptides in adjuvants.

ii.   Mixture of synthetic peptides loaded onto dendritic cells.

iii.   Recombinant protein, synthesized in - and purified

30   from - E.coli, consisting of a string bead arrangement of peptide-epitopes which are separated from each other by proteolytic cleavage sites. Protein administered in adjuvants.

iv.   Recombinant protein (see iii; mannosylated) loaded

35   onto dendritic cells.

v.   Recombinant DNA construct (naked DNA) that encodes string-beads of peptide epitopes which are

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separated by proteolytic cleavage sites.

vi.   Recombinant Canary pox virus that encodes string-
        beads of peptide epitopes which are separated by
        proteolytic cleavage sites.

5 vii.   Recombinant human adenovirus that encodes string-
        beads of peptide epitopes which are separated by
        proteolytic cleavage sites.

        Efficacy of the various vaccination protocols is assayed
10 by restimulation in mixed lymphocyte cultures of spleen cells
    of the immunized animals with autologous LPS B-cell blasts
    that are loaded with the relevant peptide(s), followed by
    measurement of the reactivity of the resulting T cell
    cultures against target cells that either present synthetic
15 peptides or the naturally processed epitopes.
        The peptide epitopes are also used for the induction of
    antigen-specific T cell activity in HLA-transgenic mixed
    lymphocyte cultures in vitro. To that end, the peptide(s) of
    choice are loaded onto either syngeneic LPS B-cell blasts or
20 dendritic cells. These cells are irradiated and used as
    stimulator cells with nylonwool passed spleen cells of HLA-
    transgenic mice. After in vitro stimulation for one or two
    weeks, the reactivity of the resulting T cell populations can
    be measured against target cells that either present
25 synthetic peptides or the naturally processed epitopes.
        Rational design of vaccines has clear advantages. Safety
    is one. For example DNA or viral vector vaccines for HPV16 E6
    and E7 are intrinsically unsafe if such vaccines contain
    functional oncogenes, but safe if the DNA or viral vector
30 encodes string beads of epitopes, a preferred embodiment of
    the present invention. Additional advantages are
    effectiveness and simplicity. Only effectively immunizing
    components are included. Irrelevant sequences are deleted,
    easing manufacture and standardization, enhancing stability
35 and decreasing cost.
        The design of effective T cell epitope vaccines hinges
    on the accurate selection of immunogenic peptides. By means

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of the current invention.(a method that analyzes the
stability of peptide-MHC complexes at the surface of antigen-
presenting cells) we have considerably improved the selection
procedure. Moreover, we have also significantly improved the
5   procedure by which poly- T cell-epitope-containing vaccines
induce strong anti-tumor and anti-viral immune responses.
        The present invention thus provides a method for the
selection of T cell peptide epitopes present in polypeptide
antigens comprising identification of peptides in the primary
10   sequence of the antigen having a binding motif and size for
binding to a HLA class I molecule, measuring the binding of
said identified peptides to MHC class I molecules, whereby
the stability of the complex of the peptide and the MHC class
I molecule is measured on intact cells carrying said MHC
15   class I molecule at their surfaces. Moreover, the present
invention provides a new method for the application of
identified T cell epitopes comprising incorporation of a
multitude of T cell epitopes in a string-of-bead construct,
in which the T cell epitopes preferably are linked to each
20   other by a spacer-sequence that ensures efficient processing
and presentation of the relevant T cell epitopes.

§3 Summary of the invention
        Peptide-binding to MHC under physiological conditions is
25   governed by dynamic balance between association and
dissociation of the MHC-peptide complexes. Both the capacity
of a peptide to bind to an MHC molecule and the stability of
the resulting MHC-peptide complex over time will determine
the amount of a given peptide-MHC complex at the surface of a
30   target/stimulator cells and, thereby, the chance that this
configuration will be detected by responding T lymphocytes.
Several assays have been set up to measure these parameters
in the context of the human and the murine immune system.
These assays are especially suitable for measuring peptide
35   binding and stability of peptide-MHC complexes in the context
of various HLA class I molecules:

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i.   The T2-assay, that measures binding of peptides to empty MHC class I MHC molecules at the surface of the processing defective cell line 174CEM.T2 (T2) (2).

ii.   An assay that measures binding of peptides to soluble class I MHC molecules that have been isolated from appropriate cell lines (6).

iii. An assay that measures binding of peptides to soluble class I MHC molecules that have been isolated as recombinant proteins from E. coli cultures that overexpress these proteins (7, 8).

iv.   An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells (3) (Example 1 herein).

v. An HLA class I peptide-binding assay that measures the stability of class I MHC-peptide complexes on intact human B cells (a major object of the present invention; see Examples 2 and 3 herein).

In one important embodiment of the present invention the latter assay is provided: a novel assay that measures MHC-peptide complex stability on intact human B cells. The binding affinity of peptides to MHC molecules under physiological conditions is a dynamic equilibrium between association and dissociation of the tri-molecular complex of peptide, MHC class I heavy chain and ß2-microglobulin. Currently, the affinity of peptides for a given class I molecule is based on assays that employ either cell-bound MHC class I molecules or purified "cell-free" MHC class I molecules. Affinity is measured by comparative capacity of peptides to upregulate MHC class I molecules on the surface of processing defective cells (2) or by their ability to compete with high affinity reference peptides (3, 9, 10). However, these assays hardly take into account the stability of peptide-MHC complexes under physiological conditions due to short incubation time, continuous presence of high concentrations of exogenous peptide, or reduced temperature. Recently, we have measured the fate of existing MHC-peptide complexes over time under more physiological conditions.

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Peptides displaying comparable binding affinities as measured
in previous assays, showed marked differences with respect to
the stability of the peptide-MHC complexes. Moreover,
stability of the peptide-MHC complex correlated better with
5   the immunogenicity of the peptide than binding affinity (see
Examples 2 and 3 of this patent application).
        Examples of self peptides displaying low binding
affinity that represent immunogenic T cell epitopes are
peptides derived from MART-1 (AAGIGILTV / ILTVILGVL) (11),
10 Pmel17/gp100 (YLEPGPVTA) (12) and p53 (LLGRNSFEV) (13). These
peptides were enclosed in this category based on results
obtained in classical binding assays. However, measurement of
the stability of the relevant peptide-MHC complexes revealed
that the stability of these complexes is comparable to that
15 of known epitopes of viral origin (see Examples 2 and 3 of
this patent application). Therefore, these peptides, although
somewhat sluggish in mounting the MHC molecule, should not be
regarded as displaying a low affinity for the presenting MHC
molecule. This notion confirms that, in addition to peptide
20 binding affinity, stability of the peptide-MHC complex is an
important parameter for the identification of immunogenic
peptide-epitopes. As MHC-peptide complex stability correlates
even better with the immunogenicity of the peptide than
binding affinity, assays measuring complex stability
25 represent an important and indispensable new step in the
sequence of procedures that is used to identify immunogenic
peptide epitopes from primary amino acid sequences.
        Another important embodiment of the present invention is
provided by the innovative method that induces T cell
30 reactivity against multiple pre-selected T cell epitopes by
immunization with a recombinant adenovirus (rAd) vector that
contains multipe T cell epitopes in a string-of-bead fashion
in which the T cell epitopes are linked to each other by
proteolytic cleavage sites. The linkage of T cell epitopes by
35 spacer sequences ensures that the T cell epitopes are
efficiently processed and presented to T cell. Therefore, the
incorporation of multiple T cell epitopes spaced by linker-

sequences preferably into recombinant adenovectors represents
an important and powerful new approach for the induction of
strong anti-viral and anti-tumor T cell immunity that is
directed against multiple T cell targets.

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§4 Detailed description of the invention

Herein we describe an assay that measures the stability
of peptide-MHC complexes at the surface of human B cell
lines. This assay, which is used to identify immunogenic
10   peptide-epitopes, constitutes a major step forward on the
road towards rational vaccine design. The novel methodology
described herein is based on a binding assay that measures
peptide binding on intact human B cells. This assay is,
therefore, separately described in the following paragraph
15   (§4.1). This binding assay has been published previously. The
stability assay, which utilizes an innovative combination of
steps is subsequently described (§4.2). The part of the
patent application describing a vaccination strategy using
rAd harbouring string-of-bead constructs encoding several
20   pre-selected T cell epitopes is given in § 4.5.

§4.1 An HLA ·class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells.

25   Peptide-binding assays employ either cell-bound MHC
class I molecules (2, 3) or purified "cell-free" MHC class I
molecules (6). Assays relying on cell-bound MHC class I
molecules are based on upregulation (2) or reconstitution of
MHC class I molecules (3) as detected by MHC class I
30   conformation-specific antibodies. Cell-free systems are
quantitative and make use of purified MHC molecules to which
labeled reference peptides are bound in a competition set-up
(6). Purification of MHC class I molecules, however, is
laborious and conformational changes may occur during
35   purification and/or storage. The peptide binding assay we use
to identify peptides which bind to various HLA class I
molecules utilizes fluorescein-labeled reference peptides

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that bind to HLA class I molecules on HLA-homozygous B-cell
lines, of which the bound peptides have been removed by mild-
acid treatment. The use of intact human B cells as tools in
peptide binding assays has several clear advantages:

5   * EBV-transformed B cells can be easily grown to high numbers
without the use of exclusive (=expensive) tissue culture
media or growth factors.
* EBV-transformed B cells express high levels of class I MHC;
no treatment with lymphokines such as IFNγ is needed to reach

10   these high expression levels.
* the mild-acid treated B cells are easily prepared;
including harvesting of the B cells from cultures, the amount
of stripped B cells needed for an average assay will be ready
for use within 30 minutes.

15   * an almost infinite repertoire of EBV-transformed human B
cell lines, expressing various combinations of class I MHC
molecules, is available in many laboratories throughout the
world
* when necessary, new EBV-transformed B cell lines can

20   readily be made within one month. EBV-transformation of human
B cells is a very straightforward procedure that is routinely
performed in many laboratories throughout the world.
        We have shown that the binding of fluorescein-labeled
peptides to these peptide-stripped HLA class I molecules is

25   specific and allows the semi-quantitative determination of
the binding-capacity of peptides. The kinetics of peptide-
binding to these peptide-stripped HLA class I molecules is
comparable to that of soluble HLA class I molecules and
independent of biosynthesis of new HLA class I molecules.

30   This assay was optimized and validated with peptides of known
binding capacity to either HLA-A*0101, HLA-A*0201, HLA-
A*0301, HLA-A*1101 or HLA-A*2401 (3, 6) (our additional
unpublished data). Furthermore, this assay was among others
applied in the identification of potential HLA-A0301-

35   restricted conserved CTL epitopes derived from HIV-1
polymerase (3).

Example 1.

Validation of a peptide-binding assay employing the HLA-A0201 and A0301 molecules on intact human B cells (adapted from (3)).

5

Materials and methods

Cell lines

The EBV transformed B cell lines (B-LCL) used for the competition assays are *JY* (HLA type: A*0201, B7, Cw7, DR4, DRw6, DPw2) and *EKR* (HLA type: A3, B7, DR7, DQw2).
The B-LCL used to confirm specific binding of reference peptides are *B109* , *BRM, D100, D110, K97, ML, NL, P98, S59* and *S99.* The HLA type of these cell lines is given in fig. 1.

15

Peptides

Fluorescein (FL)-labeled reference peptides were synthesized as Cys-derivative. Labeling was performed with 4-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs,
20   Switzerland) at pH 7.5 (Na-phospate in water/acetonitrile 1:1). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 RP-HPLC. Labeled peptides were characterized by MALDI-MS (Lasermat, Finnigan, UK). The reference peptide used for HLA-A*0301 binding was
25   KVFPC(FL)ALINK ($MH^+calc=1521.8$, $MH^+meas=1521.4$), the reference peptide for HLA-A*0201 was FLPSDC(FL)FPSV ($MH^+calc=1500.6$, $MH^+meas=1500.1$).

The reference peptides used for binding to HLA-A*0301 or HLA-A*0201 were published by Sette et al. (14). In both
30   peptides these investigators introduced a tyrosine which they used to tag a radioactive label to the peptide. We have substituted this tyrosine for a cysteine. The cysteine allowed the conjugation of 4-(iodoacetamido)fluorescein.

The polymerase amino-acid sequences of 14 different full
35   length sequenced HIV-1 virus strains: LAI, MN, NL43, OYI, SF2, RF, MAL, D31, CAM1, HAN, ELI, NDK, JRCSF and JRFL (15) were screened for possible HLA-A*0301 restricted CTL epitopes

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using a scoring system (1). The HLA-A*0301 motif used was
based on the studies of Kubo et al. (16) and Engelhard (17).
At the anchor at position 2 a L, I, V or M and at the C-
terminal anchor a K, R or Y was preferred. Peptides were
5   synthesized that contained the mentioned residues at both
anchor positions and were completely conserved among all 14
HIV-1 strains.

Peptides were synthesized by solid-phase strategies on
an automated multiple peptide synthesizer (Abimed AMS 422,
10   Langenfeld, Germany) using Fmoc-chemistry. Peptides were
analyzed by reverse phase HPLC, dissolved in 20 μl dimethyl
sulfoxide (DMSO), diluted in 0.9% NaCl to a peptide
concentration of 5 mg/ml and stored at -20°C before usage.

15   *Mild-acid treatment of B-LCL*

Mild-acid treatment of HLA-A2 or HLA-A3 on B-LCL was
performed according to Bremers modification (18) of the
procedure of Storkus et al. (19). Briefly, cells were washed
twice with PBS and then put to rest on ice for 5 minutes. The
20   cells were then treated 90 seconds with ice-cold citric-acid-
Na_2HPO_4 buffer (mixture of an equal volume of 0.263 M citric
acid and 0.123 M Na_2HPO_4) (20). For HLA-A3 the buffer was
adjusted to pH=2.9 and to pH=3.2 for HLA-A2, these pH
differences are essential for optimal elution of bound
25   peptides and reconstitution of the MHC class I molecule with
the exogenous added peptide (18). Immediately thereafter the
eluted cells were buffered with cold ISCOVE's modified
Dulbecco's medium (IMDM), washed with IMDM and resuspended at
700.000 cells/ml in IMDM + 1,5 Ug/ml ß2m (Sigma,St. Louis,
30   USA).

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Peptide competition assay

For competition assays, 25 μl FL-labeled reference peptide (end conc:150 nM in PBS) was incubated with 25 μl competitor peptide (different end concentrations in PBS) in a
5   96-well U-bottom plate (Costar, Cambridge, Massachusetts, USA). 100 μl of the mild-acid treated B-LCL (A2:JY, A3:EKR) was added to these wells.

The mixture was incubated for 3 or 24 hr at 4°C or 26°C, washed twice with PBS containing 1% BSA (PBA1%), resuspended
10   in PBA1% containing 0.5% paraformaldehyde and analyzed at a FACscan (Becton-Dickinson, Etten-Leur, the Netherlands).

The mean-fluorescence (MF) value obtained in the experiment without competitor peptide was regarded as maximal binding and equated to 0% inhibition, the MF obtained from
15   the experiment without reference peptide was equated to 100% inhibition.

% inhibition of binding was calculated using the following formula:

(1- (MF 150nM reference & competitor peptide - MF no
20   reference peptide) - (MF 150nM reference - MF no reference peptide)) x 100%

In experiments where no competitor peptide was added the fluorescence index (FI) was calculated to indicate how much
25   fluorescence above the background (no reference peptide) was measured. The FI = (MF sample - MF background)/ MF background.

To block protein synthesis in B-LCL a final concentration of 100 μM emetine (Sigma, St Louis, USA) was
30   used, as shown previously (20).

Results

Sensitivity and specificity of FL-labeled reference peptides binding to HLA class I

35   The reference peptides binding to HLA-A*0201 and HLA-A*0301 were described and used in a molecular binding assay by Sette et al. (14) In both peptides a tyrosine was used to

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tag a radioactive label to the peptide. We substituted this
tyrosine with cysteine, to which 4-(iodoacetamido)fluorescein
was conjugated.

The amount of fluorescent peptide needed for the
5   competition assay was established. For this purpose a peptide
titration was performed. After incubation of three hours at
26°C the mean fluorescence (MF) was measured. At
concentrations from 2 nM to 100 nM a sharp increase in MF was
found for the HLA-A*0201 reference peptide and from 2 nM to
10   150 nM for the HLA-A*0301 reference peptide (data not shown).
Mild-acid treatment of the B-cells before incubation with FL-
labeled reference peptide resulted in a higher fluorescence
maximum and also sharper increase of the MF at low peptide
concentrations (fig. 7).

15   In order to investigate if aspecific peptide-binding to
cell components, including other HLA class I alleles, at the
surface of the cell line used occured, 10 different B-LCL
cell lines were incubated with 0 or 150 nM of FL-labeled
reference (either HLA-A*0201 or HLA-A*0301) peptide. The FI
20   for each cell line was calculated and the FIs obtained for
reference cell lines JY (binding of peptide to HLA-A*0201)
and EKR (binding of peptide to HLA-A*0301) were equated to
100% binding. To relate the binding of FL-labeled reference
peptide to the 10 different cell lines with the binding of
25   the FL-labeled reference peptide to JY or EKR, the relative
peptide-binding percentages were determined. The relative
peptide-binding percentages of the FL-labeled reference
peptides to each cell line were calculated as: (FI cell line/
FI reference cell line)x 100%. For both FL-labeled reference
30   peptides the non-specific binding to other cell components,
of the cell lines used in the competition assay, never
exceeded 20% (fig 6). Because the peptide binding motif of
HLA-A*0301 is very similar to the binding motif of HLA-A11
(16), binding of the HLA-A*0301 FL-labeled reference peptide
35   to B-LCL cell lines expressing this allele was also observed
(fig 6). The cell line NL binds the HLA-A*0301 FL-labeled
reference peptide. It expresses the HLA-A28 allele of which

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two subtypes, HLA-Aw6801 and HLA-Aw6803, share the peptide-
binding motif with HLA-A*0301 [A. Sette, personal
communication].

5   *Kinetics of peptide-binding to mild-acid treated HLA class I
     molecules*
            To study the effect of peptide binding at different
     temperatures, EKR cells were eluted and incubated with
     FL-labeled peptide for different periods of time at 4°C,
10   26°C or 37°C, respectively. At 4°C the peptide binds
     initially rapidly and then increases steadily in time (fig.
     7). Peptide-binding at 26°C is faster (fig. 7). The amount of
     peptide bound after 6 hours at 26°C did not differ from the
     amount of peptide bound at 4°C. Peptide binds fast at 37°C
15   but no increase of bound peptide is found when incubated
     longer (fig. 7). The lack of increase in bound peptide at
     37°C is probably due to two phenomena. The HLA class I
     molecules, present on the surface of the cell to which no
     peptide was bound, desintegrate at this temperature (21).
20   Secondly, the dissociation of peptides is dramatically faster
     at 37°C compared to the dissociation of peptides when
     incubated at 4°C (22).

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Binding to mild-acid treated class I molecules is not dependent on de novo protein synthesis

To characterize the interaction of peptides with cell-associated mild-acid treated HLA molecules, peptide stripped

5   EKR cells were incubated with FL-labeled peptide for
different periods of time at 4°C or 26°C. As shown in figure
7, the fluorescent labeling at 4°C of the cells steadily
increases in time. The use of 100 μM protein synthesis
inhibitor emetine for 1 hour prior to elution decreased the

10   amount of peptide bound at 26°C but not at 4°C (fig 7).

Thus, the binding of a peptide to mild-acid treated HLA
class I molecules at 4°C was unaffected by the use of a
protein synthesis-inhibiting drug. Since metabolic processes
are reduced at 4°C, the binding of peptides to the eluted HLA

15   class I molecules is only dependent on the availability of
the HLA class I molecules already present at the outer
surface of the cell.

Competition assay

20   Plotting MF against the concentration of FL-labeled
reference peptides resulted in a log-shaped curve. We chose
150 nM of FL-labeled reference peptide as standard
concentration in all competition experiments. The use of 150
nM FL-labeled reference peptide resulted in a MF of about 4-5

25   times the background (not shown). The non-labeled reference
peptide was titrated into 150 nM of FL-labeled reference
peptide, the percentage inhibition was calculated and plotted
against the concentration of the unlabeled peptide (fig 5).
In a 24 hour competition assay at 4°C the non-labeled

30   HLA-A*0201 or HLA-A*0301 reference peptide needed about 3-5
times (respectively 0,4 μM and 0,7 μM) the concentration used
of the FL-labeled reference peptide to inhibit binding of the
FL-labeled peptide to 50% (IC_{50}) (Table 1).

To determine the optimal experimental conditions and to

35   validate the assay we tested peptides derived from HPV16 E6
and E7 proteins with known binding properties to HLA-A*0201
or HLA-A*0301 (6, 10) at different concentrations, for 3 or

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24 hours at 4°C or 26°C (Table 1). When the cells were
incubated for 24 hours less peptide was needed (Table 1). The
lowest amount of competitor peptide was needed when the cells
were incubated for 24 hours at 4°C (Table 1). No difference
5   was observed between an incubation time of 24 hours or 48
hours at 4°C (not shown). This implicates that the test is
more sensitive when equilibrium is reached. Probably, due to
a faster association of the FL-labeled reference peptide,
more competitor peptide is needed to reach IC_{50} in short
10 incubations. Ranking the peptides to their IC_{50} shows that
when the cells are incubated at 4°C for 24 hours, their order
is comparable to that found by Kast et al. (6) using the
molecular binding assay (Table 1). All peptides that did not
possess the described binding motif showed low binding
15 affinity. Taken together these results and the results of
peptide-binding to HLA class I molecules on emetine-treated
cells, we conclude that the competition assay is best
performed at 4°C with an incubation time of at least 24
hours.
20

Competition with known CTL epitopes

   Five HLA-A*0201 restricted CTL epitopes, one HLA-A*0301
restricted CTL epitope and two HLA-A*0301 peptides,
identified via peptide pool-sequencing, were used to
25 determine the IC_{50}-values of high affinity binding peptides.
The five peptides tested for binding to HLA-A*0201 all
competed very well with an $IC_{50} \leq 1.7$ μM (Table 2). The known
HLA-A*0301 restricted CTL epitope derived from HIV was
tested. This peptide, derived from HIV-nef, bound with an
30 IC_{50} of 0.5 μM. The two peptides, which were identified via
peptide pool sequencing bound with an $IC_{50} \leq 15$ μM (Table 2).
We therefore conclude that peptides competing with an IC_{50}
≤ 5 μM must be considered potential CTL epitopes.

35 *Binding of conserved HIV-1 pol sequences to HLA-A*0301*

   Twenty peptides of 8-11 amino acids long were selected
on the basis of the HLA-A*0301 binding motif and their

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conservation in the polymerase gene products of different
HIV-1 strains. The peptides were tested in the competition
assay for 24 hours at 4°C. Nine peptides were shown to bind
to HLA-A*0301. Four peptides bind with intermediate-affinity
5   and competed with an $IC_{50} \leq 5$ μM (Table 3), the other five
peptides (marked with an asterisk; *) bind with high affinity
and competed with an $IC_{50} \leq 3.0$ μM. Considering the IC_{50}
obtained with the known CTL epitopes, especially these five
peptides may be candidate CTL epitopes.
10

Comments

     For an extensive discussion of these results see (3).
This example shows that this assay performs well with respect
to peptides of known binding capacity to either HLA-A*0201 or
15   HLA-A*0301. The kinetics of peptide binding in this assay
were shown to be comparable to that in assays employing
soluble HLA class I molecules. Furthermore, application of
the assay in the search for potential HLA-A*0301 restricted
CTL epitopes, derived from HIV-1 polymerase, resulted in the
20   identification of five high-affinity binding peptides. The
assay is easy to perform because there is no need to purify
HLA class I molecules, or to transfect cells with HLA class I
molecules and no radioactive label is used. Moreover, large
panels of HLA-typed human B-cell lines are available, as
25   tools for peptide-binding to a vast array of HLA molecules.
Presently, the system is also used succesfully for the
identification of peptides that bind to HLA-A*0101 and HLA-
B7.

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<u>Legends to Figures Example 1</u>

Figure 1 . Specificity of FL-labeled reference peptides.

Reference cell line EKR (HLA-A*0301) was mild-acid
treated at pH=2.9. The reference cell line JY (HLA-A*0201)
5   was mild-acid treated at pH=3.2, and the 10 different other
B-LCL lines were mild-acid treated at pH=2.9, when subjected
to incubation with the HLA-A*0301 FL-labeled reference
peptide, or at pH=3.2 when incubated with the HLA-A*0201
FL-labeled reference peptide. EKR cells are incubated with
10 150 nM of the HLA-A*0301 FL-labeled reference peptide (*open
bars*), JY cells are incubated with 150 nM of the HLA-A*0201
FL-labeled reference peptide (*hatched bars*) and the 10
different other B-LCL lines were incubated with 150 nM of
either the HLA-A*0301 (*open bars*) or HLA-A*0201 FL-labeled
15 reference peptide (*hatched bars*), for 4 hr at 26°C. The
fluorescence index (FI) was calculated for each cell line and
the FI of FL-labeled reference peptide bound to EKR (for
binding to HLA-A*0301) and the FI of FL-labeled reference
peptide to JY (for binding to HLA-A*0201) was equated to 100%
20 binding. By the formula: (FI cell line/FI reference cell
line)*100% the relative peptide-binding percentages of the 10
different B-LCL lines was calculated. The upper left side
shows the full HLA-type of the reference cell lines together
with the overlapping HLA-type of other cell lines. The lower
25 left side shows all 10 B-LCL lines with their full HLA-type.

*Figure 2 . Peptide binding on eluted vs not-eluted HLA class
I molecules.*

JY cells (*closed symbols*) and JY cells of which their
30 HLA class I molecules were mild-acid treated (*open symbols*),
were incubated with increasing amounts (nM) of the HLA-A*0201
FL-labeled peptide. Cells were incubated for 3 hours at 26°C,
washed and mean-fluorescence (mF) was measured at a FACScan.
The lines shown are the result of logarithmic regression
35 analysis of the concentration of FL-labeled reference peptide
versus the mF.

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Figure 3 . Kinetics of peptide binding to mild acid treated HLA class I molecules.

EKR cells were mild acid treated and incubated with 150 nM of HLA-A*0301 FL-labeled reference peptide for different
5   periods of time at 4°C (*triangles*), 26°C (*open squares*) or 37°C (*closed squares*). At 10, 20, 40, 90, 180 and 360 minutes the binding of Fl-labeled peptide was measured. Binding is given as the fluorescence index (FI). The lines shown for 4°C and 26°C are the result of respectively lineair or
10   logarithmic regression analysis.

Figure 4. Binding of FL-labeled peptide to protein synthesis inhibiting drug treated cells.

EKR cells were treated with 10^{-4}M emetine (*open bars*) or
15   not (*hatched bars*), for 1 hour prior to mild-acid treatment (20). 150 nM of HLA-A*0301 FL-labeled reference peptide was added and binding was monitored at 1, 3 or 4.5 hours of incubation. Cells were incubated at 26°C or 4°C.

20   *Figure 5. Competition of non-labeled reference peptide with FL-labeled reference peptide.*

EKR cells (left) or JY cells (right) were incubated with 150 nM of FL-labeled reference peptide, kvfpC(FL)alink or flpsdC(FL)fpsv respectively, and increasing amounts (µM) of
25   non-labeled reference peptide. Inhibition of binding was calculated and showed in relation to the amount of non-labeled reference peptide used.

§4.2 An HLA class I peptide-binding assay that
30   **measures the stability of peptide-MHC complexes at the surface of intact human B cells.**

The binding affinity of peptides to MHC molecules at equilibrium is the resultant of the continued association and dissociation of the tri-molecular complex of peptide, MHC
35   class I molecule and ß2m. The dissociation rate of peptides bound to MHC class I, is neither influenced by the presence of competing peptides (23) nor by the concentration of the

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competing peptides (24). On the other hand, the amount of
free MHC peptide binding sites is influenced and limited by
the dissociation rate of previously bound peptide (24). Thus
a peptide with a low dissociation rate will, once bound,
5   probably form a stable MHC-peptide complex in the ER, be
transported to the cell-surface and persist there for a time
sufficient to allow T-cell recognition.

    In order to investigate the correlation between
stability of the peptide-MHC complex and immunogenicity we
10   have determined the dissociation rate of a group of MHC class
I binding peptides. This assay measures the stability of
peptide-MHC complexes at the surface of intact HLA-homozygous
B-cells. Comparison of the correlation between immunogenicity
and peptide binding affinity on one hand and between
15   immunogenicity and the dissociation rate of peptide from MHC
class I molecules on the other hand has shown that
immunogenicity correlates better with the dissociation rate
than with peptide binding affinity.

    As this complex-stability assay makes use of intact
20   human B cells, it shares the advantages described for the
peptide binding affinity assay (see §4.1).

Example 2.
Immunogenicity of peptides bound to MHC class I MHC
25   **molecules correlates well with stability of the MHC-
peptide complex.**

<u>Material and Methods</u>
Cell lines
30   The EBV transformed B-cell line: JY (HLA type:A*0201,
B7, Cw7, DR4, DRw6, DPw2) was cultured in complete culture
medium consisting of RPMI 1640 Dutch modification (Gibco BRL,
Paisley, Scotland) supplemented with 10% FCS, antibiotics
(100 IU/ml penicillin (Brocades Pharma, Leiderdorp, The
35   Netherlands) and 100 ug/ml kanamycin (Sigma, St. Louis, MO,
USA)), and 20 μM 2-ME (Merck, Darmstadt, Germany) at 37°C in
humidified air containing 5% CO_2.

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Jurkat A*0201K^b cells are stable transfectants of the
human T cell leukaemia line, Jurkat, which express the
product of the HLA-A*0201K^b chimeric gene (25). They are
cultured in complete culture medium in the presence of
5 200ug/ml G418 sulphate.

Peptides

Peptides were synthesized by solid-phase strategies on
an automated multiple peptide synthesizer (Abimed AMS 422,
10 Langenfeld, Germany) using Fmoc-chemistry. Peptides were
analyzed by reverse phase HPLC, dissolved in 20 μl DMSO,
diluted in 0.9% NaCl to a peptide concentration of 5 mg/ml
and stored at -20°C before usage.

Fluorescein (FL)-labeled peptides as used in the
15 competition based HLA class I binding-assay were
synthesized, labeled and characterized as described earlier
(3). The sequence of the reference peptide used for HLA-
A*0201 was FLPSDYFPSV (14) wherein we substituted the
tyrosine with a cysteine to tag a fluorescein group to the
20 peptide: FLPSDC(FL)FPSV (3).

Transgenic mice

HLA-A*0201K^b transgenic mice were kindly provided by
Dr L. Sherman (Scripps Laboratories, San Diego, USA; through
25 animal distributor Harlan Sprague Dawley, Inc., Indianapolis,
USA). Mice were held under clean conventional conditions. The
transgenic mice express the product of the HLA-A*0201K^b
chimeric gene in which the a3 domain of the heavy chain is
replaced by the corresponding murine H-2 K^b domain while
30 leaving the HLA-A*0201 a1 and a2 domains unaffected (25).
This allows the murine CD8 molecule on the murine CD8+ T
cells to interact with the syngeneic a3 domain of the hybrid
MHC class-I molecule.

In vivo immunizations and murine T cell cultures

Groups of 3-6 HLA-A*0201K^b transgenic mice were injected subcutaneously in the base of the tail with 100ug peptide emulsified in IFA in the presence of 140ug of the H-2 I-A^b-

5   restricted HBV core antigen-derived T helper epitope (128-140; sequence TPPAYRPPNAPIL) (26). After 11 days, mice were sacrificed and spleen cells (30x10^6 cells in 10 ml) were restimulated *in vitro* with syngeneic irradiated LPS-stimulated B cell lymphoblasts (ratio 3:1), and 1 ug/ml

10 peptide in complete culture medium in T25 flasks (Falcon, New Jersey, USA). At day 6 of culture, the cytotoxicity of these bulks was tested in a standard 5 hour ^{51}Chromium (^{51}Cr) release assay.

15 *^{51}Cr Cytotoxicity assay*

CTL activity was measured in a standard chromium release assay as described previously (27). Target cells were sensitized with 10 ug/ml peptide for 30' at 37°C. Target cells were added to various numbers of effector cells in a

20 final volume of 100 μl of complete culture medium in 96-wells U-bottom microtiter plates. After 5 hours of incubation at 37°C, supernatants were harvested. The mean percentage specific lysis of triplicate wells was calculated as follows:
% specific lysis = ((experimental release-spontaneous

25 release) / (maximal release-spontaneous release)) x 100
Percentage specific lysis is expressed in
LU30%/10^{6}cells, in which 1 LU30% corresponds to the number of effector cells required to induce 30% ^{51}Cr release from 2000 Jurkat A*0201/K^b target cells during a 5-h assay.

30

Peptide 'stripping' by mild-acid treatment and competition based HLA class I peptide-binding assay
See Example 1

35 *Measurement of MHC-peptide complex stability at 37°C*

JY cells at a concentration of 1-2 million cells/ml were incubated with 10^{-4}M emetine (Sigma, St. Louis, USA) for 1

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hour at 37°C to stop protein synthesis and thus the emergence
of *de novo* synthesized class I molecules at the cell-surface
(20). Cells were washed twice with PBS and peptide-stripped
(see above). One million cells were added to 200ug peptide in
5   1 ml and incubated for 1 hour at room temperature. Cells were
washed twice with ice-cold IMDM and resuspended in 1 ml IMDM.
Subsequently, the cells were incubated for 0, 2, 4 and 6
hours at 37°C and thereafter stained with BB7.2, an HLA-A2
conformation specific monoclonal antibody (28) and GaM/FITC.
10 Thereafter the cells were fixed by resuspension in PBA1%
containing 0.5% paraformaldehyde. Cells were analyzed by
FACscan. The fluorescence index (FI) was calculated as FI=
(mean fluorescence sample - mean fluorescence background) /
mean fluorescence background (without peptide). Samples were
15 tested in duplo and the variation between both samples was
allways less that 10%.

      The percentage of residual HLA-A2 molecules was
calculated by equating for each peptide, the FI of t=0 to
100% and then use the formula: %remaining= $(FI_{t=n} / FI_{t=0})$ x
20 100%. As the dissociation of peptides from MHC is a linear
process, the stability of the peptide-MHC complexes was
measured as the time required for 50% of the molecules to
decay (DT50%). We've used t=2 hours at 37°C as starting point
for the reason that from this time point only the DT50% are
25 determined from peptides that are able to form stable
peptide-MHC complexes.

Statistics

      Using the Fisher's test for 2 by 2 tables (Fisher's
30 exact 2-tailed test), the dissociation rate (DT50%) of
peptides at 37°C was correlated to the immunogenicity of the
peptides. Binding-affinity could not be correlated to
immunogenicity using a Chi-square test due to the relatively
small number of peptides. Therefore we compared high affinity
35 binding peptides with low affinity binding peptides in order
to establish the strongest correlation between affinity and
immunogenicity using the Fisher's test for 2 by 2 tables.

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<u>Results</u>

*Stability of MHC class-I molecules complexed with HBV or
HPV16 derived peptides of known binding affinity and
immunogenicity in HLA-A*0201/K^b transgenic mice*

5   To study the relation between dissociation of peptides
bound to MHC class-I molecules and their ability to induce a
CTL response, we used 9 peptides derived from HBV polymerase
(pol) and 8 peptides of HPV16 of which the relative binding
affinity and immunogenicity in HLA-A*0201/K^b transgenic mice

10   was reported previously (6, 10, 29). To show that all 17
peptides indeed bound to HLA-A*0201 we tested their affinity
in a previously described competition based HLA-class I
binding-assay (3). HBV*pol*-635, HPV16*E7*-11 and HPV16*E7*-86
bound with relatively high affinity (< 5 µM). Fourteen

15   peptides bound with intermediate (between 5 and 15 µM) or low
affinity (> 15 µM; Table I). Peptide binding affinities
measured and classification of the peptide binding affinity
into high, intermediate and low are comparable to the
affinities and classifications of Sette *et al.* (10) and Kast

20   *et al.* (6).

Subsequently with the use of a conformation specific
anti-HLA-A2 antibody, the amount of residual HLA-A*0201
peptide complexes was monitored in time. The loss of peptide-
stabilized HLA-A*0201 molecules at the cell-surface

25   represents the dissociation of the peptide from the class-I
molecule to which the peptide is bound. The stability is then
presented by the time required for 50% of the molecules to
decay (DT50%). All three high affinity binding peptides and
three of the intermediate affinity binding peptides, HBV*pol*-

30   996, HBV*pol*-1076 and HPV16*E7*-82 showed a DT50% of more than 3
hours (Table I). The four other peptides of intermediate
affinity, HBV*pol*-1344, HPV16*E6*-18, HPV16*E6*-52 and HPV16*E7*-7
showed a DT50% between 1 and 2 hours (Table I). The low
affinity binding peptides showed a DT50% of 1 hour or less.

35   In Table II we show a comparison between the dissociation
rate, binding affinity and immunogenicity of these peptides.
All high affinity binding peptides form stable MHC-peptide

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complexes and are immunogenic, whereas the group of peptides
of intermediate affinity contains either peptides that are
immunogenic and form stable MHC-peptide complexes or are non-
immunogenic and do not form stable MHC-peptide complexes as
5   shown by their high dissociation rates (Table II).

*Stability of MHC class-I molecules complexed with known human
CTL epitopes*

        Seventeen HLA-A*0201 binding peptides earlier reported
10   to be immunogenic (e.g. found as CTL epitope or capable of
inducing a primary response) (6, 12, 27, 30-40) were tested
for their binding affinity to HLA-A*0201. Eight peptides
bound with high affinity, 7 peptides bound with intermediate
affinity and 2 peptides bound with low affinity (Table III).
15   The dissociation rates were determined and virtually all
peptides showed a DT50% > 4 hours, except for the peptides
HPV11*E7*-4 and HIV-1*pol*-267. The HPV11*E7*-4 and HIV-1*pol*-267
CTL epitopes, both found by primary CTL induction using
synthetic peptide or cells expressing extremely high amounts
20   of antigen, dissociated faster (DT50% > 2 hours ; Table III).
Interestingly, the sequence of the HCV1*core*-131 peptide
[ADLMGYIPLV] does not correspond precisely to the HLA-A*0201
motif. The HCV*core*-132 peptide which lacks the N-terminal
alanine [DLMGYIPLV] fits better to the HLA-A*0201 motif. This
25   is also reflected in the higher affinity of this shorter
peptide (IC_{50}=5.0 μM) but the peptide dissociates
dramatically faster (Fig 1.) than the HCV*core*-131 peptide.

Immunogenicity is correlated with the dissociation rate
30   A significant correlation exists between the
immunogenicity of a peptide and the dissociation rate. Of the
investigated known HLA-A*0201-restricted immunogenic
peptides, 21 out of 23 showed a DT50% > 3 hours, while none
of the 11 non-immunogenic peptides showed a DT50% >3 hours
35   (p=0.0000003, Table IV). This correlation is closer than that
between peptide binding affinity and immunogenicity
(p=0.0005, Table IV) and confirms the trend visible in

25

Table II. When the correlation between immunogenicity and
dissociation rate was investigated for peptides binding with
intermediate or low affinity, this was still better
correlated (p=0.00007, Table V) to immunogenicity than

5   affinity (p=0.04). This implies that peptides that are
processed, transported to the endoplasmic reticulum and are
able to form stable MHC-peptide complexes are likely to be
CTL epitopes.

10   *Immunogenicity in HLA-A*0201/K^b transgenic mice of HIV-1*
derived peptides with known affinity and dissociation rate
         To assess the in vivo immunogenicity of peptides of
which the binding affinity and the dissociation rate was
measured, HLA-A*0201/K^b transgenic mice were vaccinated with

15   two control peptides (HPV16E7-86 and HBVcore-18; FLPSDDFPSV)
and four HIV-1 derived peptides (Table VI). The derivation of
these transgenic mice (25) and their use to analyze in vivo
immunogenicity have been described previously (10, 29). The
HIV-1pol-468;(ILKEPVHGV) is a CTL epitope and binds with

20   intermediate affinity. The HIV-1pol-267;(VLDVGDAYFSV) peptide
was found to be immunogenic in a human primary CTL induction
after repetitive stimulations with relatively high doses of
peptide (27). To test the predictive value of the *in vitro*
measured MHC-peptide complex stability we determined the

25   binding-affinity and dissociation rate of the two other HIV-
1pol peptides (HIV-1pol-343: YMDDLYVGSDL and HIV-1pol-576:
LLWKGEGAV) (Table VI). Both peptides were detected when the
highly conserved regions of HIV-1pol were screened for amino
acid sequences that contained two anchors for binding to HLA-

30   A*0201, as described previously (27). We vaccinated groups of
mice with all the peptides. Bulk CTL derived from mice
vaccinated with the control peptides specifically lysed
peptide-sensitized Jurkat A*0201/K^b cells (Fig. 2; Table VI).
As expected, all peptides with a low dissociation rate

35   mounted a CTL response (Fig. 2 ; Table VI), whereas the two
peptides with high relative dissociation rates did not induce
a CTL response (Fig. 2; Table VI). Thus, the immunogenicity

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of these peptides was perfectly predicted by their dissociation rates.

Comments

5   An extensive discussion of these results with respect to HIV-specific T cell immunity will be reported elswhere (Van der Burg et al., in preparation). This example shows that the measurement of MHC-peptide complex stability is highly valuable in identifying potential T cell epitopes. Newly

10   defined immunogenic peptides formed relatively stable MHC-peptide complexes as shown by their low dissociation rates, whereas non-immunogenic peptides displayed high dissociation rates. In addition, virtually all previously described HLA-A*0201 restricted T cell epitopes showed low dissociation

15   rates. Furthermore, we show that the immunogenicity of HIV-1 derived peptides can be predicted more accurately by their dissociation rate than by the MHC class I binding affinity. We find a closer correlation between the dissociation rate of a peptide and immunogenicity ($p=0.0000003$) than between

20   binding affinity and immunogenicity ($p=0.0005$). The better correlation is gained in the group of peptides that bind with intermediate or low affinity. In conclusion, selection of peptides based on affinity and their dissociation rate leads to a more precise identification of candidate CTL epitopes

25   than selection based on affinity alone.
     Note that this assay requires HLA-type specific MAbs that can discriminate between empty and peptide-loaded molecules. Although such Abs are currently available for HLA-A*0201 and -A*0301, additional Abs need to be identified or

30   isolated for defining the stability of peptide-MHC complexes in the context of other HLA class I molecules. Approaches to isolate such Abs include:
     *   Screening of available Abs (ATCC, other laboratories) for desired characteristics

35   *   Selection of appropriate Abs against a human B cell line expression the relevant HLA-molecule from a semi-synthetic phage antibody display library (in collaboration with Dr. T.

27

Lochtenberg, University Hospital Utrecht, The Netherlands;
(41)).

*   Generation of monoclonal Abs, or selection of phage
antibodies against purified, peptide-loaded MHC molecules
5   (6).

Legends to Figures Example 2

*Figure 6. Binding affinity and dissociation rate of the
HCV1core-131 peptide and the shorter variant without the*
10 *N-terminal alanine.*

The binding affinity (left) and the dissociation rate
(right) of the HLA-A*0201 restricted CTL epitope HCV1*core*-131
[*closed symbols*; ADLMGYIPLV] (31) and shorter variant [*open
symbols*; DLMGYIPLV], which corresponds more precisely to the
15 HLA-A*0201 motif, was tested (see material & methods). The
mean inhibition of the reference peptide at each
concentration of competitor peptide, obtained in two
independent experiments, is shown at the left. The right
figure shows the percentage of residual peptide-MHC molecules
20 for both peptides at each time-point (mean of two independent
experiments). The percentage of molecules present at t=2
hours was set to 100%. The lines are the result of linear
regression analysis.

25 *Figure 7. Peptide-specific cytotoxicity induced by
vaccination of HLA-A*0201K^b transgenic mice.*

A representative experiment in which HLA-A*0201K^b
transgenic mice were vaccinated with indicated peptide
displaying a low dissociation rate (A,B,E) or high
30 dissociation rate (C,D) in combination with an HBV core-
encoded T helper epitope in IFA (see material and methods).
Bulk CTL cultures derived from spleen cell of these mice were
tested for peptide specificity in cytotoxicity assays on
Jurkat A*0201K^b target cells pulsed with (*open symbols*) or
35 without (*closed symbols*) specific peptide. Shown is the mean
specific lysis of bulk CTL from 3-6 animals with indicated

28

standard deviation. Specific lysis is depicted at E/T ratio varying from 1.5 to 100.

Example 3

5   Identification of melanoma associated immunogenic peptides using an assay that measures stability of the MHC-peptide complex.

<u>Materials and methods</u>

10   Most procedures have been described in Examples 1 and 2. Induction of CTL by stimulating human T lymphocytes with peptide-loaded dendritic cells (DC) was performed as follows: Monocyte-enriched Human Peripheral Blood Monocyte (PBMC) fractions were isolated by plastic adherence of total PBMC

15   from HLA-A*0201-subtyped healthy donors. Adherent cells were cultured for 5-7 days with RPMI/Lglutamine/antibiotics/10% FCS or 10% human serum (HS), and 500 U/ml rHuIL-4, and 800 U/ml rHuGM-CSF. Culture medium with cytokines was replenished every other day. Cultures were treated for 24 h with 50 U/ml

20   rHuIL-1a and 200 U/ml g-IFN, and pulsed with 50 ug/ml peptide in RPMI/L-glutamine/antibiotics/1% FCS for 4 h. Peptide-pulsed stimulators were irradiated (2500 Rads) and washed twice. In each well of a 24-well plate 1 ml of RPMI/L-glutamine/antibiotics/5% HS was dispensed containing 1-

25   2×10^4/ml stimulator cells.

        Autologous responder cells were enriched for (CD8+) T-cells by adherence to plastic dishes, followed by depletion of CD4+ cells using Dynabeads (Dynal, Olso, Norway). Total PBMC responders were mixed with the CD8-enriched non-adherent

30   cells, to bring the final responder population to approximately 10% CD4+ T cells. Responders were mixed with stimulators in a 1:10 to 1:20 ratio, to a total of 2×10^6 responders per well. rHuIL-7 was added to 5 ng/ml. Medium + rHuIL-7 was replenished after 7 days. At day 12, responders

35   were restimulated with autologous peptide-pulsed adherent PBMC (as described previously: (42)). rHuIL-2 was added to a

29

final concentration of 120 IU/ml. Similarly, CTL cultures
were restimulated weekly. CTL cultures were subcloned in U-
bottom 96-well plates by limiting dilution, using the HLA-
A*0201+, MelanA/MART-1 expressing FM3 Melanoma cell line
5   (5000/well; (43)), and a mixtures of allogenic PBMC from six
donors (100.000/well) and three HLA-A*0201+ B-LCL
(5000/well), in RPMI/Lglutamine/antibiotics/5% HS + 120 IU/ml
rHuIL-2. Clones were restimulated weekly.

10 Results

        The melanoma antigen Melan-A/MART-1 was screened for the
presence of potential HLA-A*0201-binding CTL epitopes using
three peptide binding assays: the T2-binding assay (2), a
binding assay that uses HLA-A*0201-molecules on intact human
15 B cells (see Example 1), and an assay that measures the
stability of the MHC-peptide complexes (see Example 2).
Comparison of the binding-data (see Table IX) shows that the
nonamer peptide AAGIGILTV, which represents a previously
described immunodominant peptide-epitope presented by HLA-
20 A*0201-positive melanoma cells (44), binds poorly to T2 cells
and only shows modest binding to HLA-A*0201 on intact human B
cells. The 10-mer variant of this peptide (EAAGIGILTV),
however, displays considerable binding to HLA-A*0201 in both
binding assays. Paradoxically, comparison of these two
25 peptides with respect to the stability of peptide-MHC
complexes shows that the 9-mer peptide, when bound to HLA-
A*0201, forms stable peptide-MHC complexes, whereas complexes
with the 10-mer peptide are unstable.
        Peptide-specific CTL immunity was raised in vitro by
30 stimulating peripheral blood lymphocytes of HLA-A*0201-
positive healthy donors with autologous dendritic cells that
were loaded with either of the two peptides. The reactivity
of the resulting CTL was tested against T2 cells loaded with
the relevant peptides as well as to HLA-A*0201- and MART-1-
35 positive human melanomas cells. These experiments clearly
demonstrated that tumor-specific CTL activity that reacted
against both peptide-loaded T2 cells and melanoma cells was

30

only obtained after stimulation of the donor lymphocytes with
the 9-mer peptide AAGIGILTV (these experiments will be
described elswhere; Van den Elsas et al., manuscript in
preparation). These data clearly demonstrate that
5   immunogenicity of a peptide epitope correlates strongly with
the stability of the corresponding peptide MHC complex,
whereas MHC-binding of a peptide as measured on T2 cells or
intact B cells does not ensure that this peptide (i) will
form stable MHC-peptide complexes and (ii) is immunogenic
10 found to show strong and stable binding to HLA-A*0201 in all
three assays (see Table IX). Also against these two peptides
CTL reacting against both peptide-loaded T2 cells and HLA-
A*0201-/MART-1 positive melanoma cells could be raised (Van
den Elsas et al., manuscript in prep.).
15   Taken together these results show that selection of
immunogenic peptides based on stability of the MHC-peptide
complex is a valuable tool in the identification of tumor-
associated T cell epitopes.

20 §4.3 Identification of immunogenic peptides
          The present invention provides an novel technique for
identifying MHC-binding peptides that can serve as a target
for an immunotherapeutical T cell response. This method will
be applied in conjunction with other selection steps (see §1)
25 to screen the primary sequence of proteins that are expressed
by for instance tumors for peptides that are likely to be
processed and presented by tumor cells and that will
constitute an immunogenic target for the T cell immune
system.
30   Peptide-epitopes derived from the following antigens are
included in our studies:
*   E6-protein of human papilloma virus type 16 and 18
(HPV16, HPV18)
*   E7-protein of human papilloma virus type 16 and 18
35 (HPV16, HPV18)
*   Gag, Pol and Env-proteins of human immunodeficiency
virus (HIV)

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* MAGE-2 human melanoma antigen
* Tyrosinase human melanoma antigen
* Melan-A/MART-1 melanoma antigen
* p21Ras human onco-protein
5 * p53 human onco-protein
* human carcino-embryonic antigen (CEA)
* human epithelial cell adhesion molecule (EpCAM)
* CD19 human B cell-specific protein
* CD20 human B cell-specific protein
10 * CD44 cell surface glycoprotein
* The immunoglobulin (Ig) variable domains of the Ig heavy
  and light chains expressed by B cell lymphomas

The sequences of the proteins mentioned above are screened
15 for peptides that are likely to represent immunogenic T cell
epitopes in the context of the following HLA class I
molecules:
* HLA-A*0101
* HLA-A*0201
20 * HLA-A*0301
* HLA-A*1101
* HLA-A*2401
* HLA-B7

25 § 4.4 List of peptides screened in a stability assay
because they have been through the preselection
procedures.

As illustrated in § 4.3 Examples 2 and 3, selection of
immunogenic peptides is greatly improved in accuracy when
30 peptides are screened not only for binding to the MHC
molecules concerned, but also for the stability of the
resulting peptide-MHC complexes. In previous publications we
have described multiple potential immunogenic peptides
derived from various (e.g.tumor-)antigens. These peptides
35 were selected on the basis of a two-step procedure,
consisting of (i) computer-prediction and (ii) binding assays
that do not take into account the stability of peptide-MHC

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complexes. The person skilled in the art can now apply, and
thereby further validate, our novel assay for measuring
peptide-MHC complex stability with respect to these peptides.
A list of these peptides is provided in Tables X - XX.

5

§ 4.5 Vaccination with recombinant adenoviruses
harbouring several defined T cell epitopes in string-
of-bead constructs.

       T cell-mediated immunity to viruses or tumors can be
10   induced in two ways: passive, by transfer of virus- or tumor
specific T cells, or active, by exposure to antigen. In the
latter case, antigen can be given to the host in many
different forms, ranging from whole attenuated viruses or
tumor cells to isolated proteins. In virtually all these
15   cases the vaccines are not rationally designed in the sense
that the minimal essential T cell epitopes are known.
Therefore, immunization in these cases may not always leas to
the desired effect. For example, immunization with attenuated
viruses, like vaccinia, may induce unwanted side-effects or
20   result in T cell immunity to epitopes that are subjected to
antigenic variation by the wild-type virus. Likewise,
immunization with a single protein can be ineffective,
because it may induce only T cell-responsiveness to the
immunodominant T cell epitopes, without inducing T cell-
25   responses to other, subdominant T cell epitopes, or it may
not contain sufficient CTL epitopes to cover the whole target
population. In part, these disadvantages can be overcome by
exploiting other vaccination strategies.
       Vaccination strategies using recombinant viruses
30   expressing the antigens of choice are currently under
development. In the case of the development of anti-tumor
vaccines, several tumor-associated antigens, like MART1 and
gp100 are good candidates for the incorporation into a
recombinant viral vector. However, the delivery of whole
35   genes encoding tumor-associated antigens by recombinant viral
vectors as a way to evoke anti-tumor immunity might be unsafe
when these tumor-associated antigens are involved in

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carcinogenesis. For example, viral vector vaccines for treatment and prevention of HPV16-positive cervical carcinoma are intrinsically hazardous if such vaccines contain the functional human papilloma virus type 16 (HPV16) E6 and E7

5   oncogenes. The same holds true when the viral vector encodes the oncoprotein HER2/neu, cyclin-dependent kinase 4, the aberrant fusion proteins BCR-ABL or mutated Ras and p53 proteins, because these genes are implicated in the development of cancer. Likewise, incorporation of the genes

10   belonging to the family of tumor-associated antigens MAGE, GAGE or BAGE into viral vectors should be avoided, because their function has until now not been identified. However, by introducing only the sequences that encode T cell epitopes derived from such tumor-associated antigens into recombinant

15   viral vectors it should be feasible to direct the immune response to those targets without introducing potential hazards as transformation of somatic, vector infected cells.
     Recently, studies have been reported that describe the successful use of a recombinant vaccinia vaccine expressing

20   several CTL epitopes in a string-of-bead fashion in mice (48), (49). These studies show the potency of the use of string-bead-vaccines for the induction of anti-viral and anti-tumor immunity. However, due to the potential risks associated with vaccinia and the decreasing or absent (in

25   younger individuals) immunity to poxvirus due to the abolished vaccination programme with poxvirus, recombinant vaccinia vaccines cannot be used in humans. Moreover, in these studies the CTL epitopes were directly linked to each other, and did not contain spacer-sequences that direct

30   efficient and accurate processing and presentation of the CTL epitopes. For these reasons, rAd harbouring several CTL epitopes in a string-of-bead fashion with proteolytic cleavage sites between the CTL epitopes leading to optimal processing and presentation of the incorporated CTL epitopes

35   will induce stronger CTL responses without inducing harmfull side-effects.

34

Recombinant adenovirus, harbouring whole tumor-
associated antigens, have been used to induce protective
anti-tumor immunity (50-52).(53, 54), illustrating the
possibility to use rAd for the induction of tumor-specific
5   protective immunity.

By incorporation of minigenes containing multiple-T
cell-epitopes and proteolytic cleavage sites in between these
T cell epitopes into a rAd we now have developed a novel and
innovative method for the induction of protective T cell
10   responses against viruses and tumors.

Example 4.
An rAd expressing several defined CTL epitopes in a
string-of-bead fashion induces protective anti-tumor
15   immunity.

<u>Materials and methods</u>

<u>Cell lines</u>
20   Mouse embryo cells (MEC), Ad5E1 transformed MEC, Ad5E1 +
ras transformed MEC, HPV16-transformed MEC, COS-7 cells were
maintained in Iscove's modified Dulbecco's medium (Biocrom
KG, seromed, Berlin, Germany) supplemented with 4% FCS
(hyclone laboratories , Logan, Utah), penicillin, (110 IU/ml;
25   Brocades Pharma, Leiderdorp, the Netherlands), and
2-mercaptoethanol (20 µM) at 37°C in a 5% CO_2 atmosphere. CTL
clones were cultured as described elsewhere (55, 56), (1057
The influenza matrix-specific HLA-A*0201-restricted CTL clone
was grown on HLA-A*0201-positive EBV-transformed B cell lines
30   irradiated with 30 Gy in RPMI. 911 cells were grown as
described in (58).

<u>Generation of rAd</u>
Minigene 1 or minigene 2 (see Fig 8) were inserted into
35   the shuttle vector pMad5. pMad5 (R. Hoeben, unpublished) was
derived from pMLP10 (73) through the following cloning steps:
(i) deletion of the SalI/BamHI-fragment, (ii) insertion of a

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polylinker sequence (ClaI, MluI, SnaBI, SpeI, AsuII, MunI)
into the unique Hind III site, directly downstream of the Ad5
major late promoter (MLP) and Ad2 tripartite leader
sequences, (iii) Insertion into the MunI site of a BglII/XhoI

5   fragment of the Ad5 genome, which permits homologous
recombination of the pMad5 sequences with sequences of pJM17
(see below). Insertion of minigenes 1 and 2 was performed in
two steps. First pMad5 was cleaved with enzymes SpeI and MluI
and the 5'ends were dephosphorylated. The annealed and

10 phosphorylated double-stranded oligonucleotides 1a/b and 2a/b
(see Table A) were ligated into this vector, which resulted
in a small open reading frame consisting of a methionine, a
spacer with the sequence NASYATS and the human c-myc sequence
SEQKLISEEDLNN. The latter sequence corresponds to an epitope

15 which can be recognized by the appropriate monoclonal
antibody (74). As a result of the cloning strategy, the
original SpeI and MluI sites of pMad5 were destroyed, whereas
new SpeI and MluI sites were created between the Start codon
and the c-myc epitope encoding sequence. In a second cloning

20 step the CTL epitope encoding sequences were inserted into
the cassette. The cassette vector was cleaved with enzymes
SpeI and MluI and the annealed non-phosphorylated double-
stranded oligonucleotides 3a/b and 4a/b were ligated into the
open vector (minigene 1). Alternatively, the annealed non-

25 phosphorylated double stranded oligonucleotides 5a/b and 4a/b
were ligated into the open vector (minigene 2). Subsequently,
the non-ligated oligonucleotides were removed from the
ligation mixture by Sephacryl 400 column-purification. The
eluted DNA was added to a ligation-reaction that contained

30 the annealed and phosphorylated double-stranded
oligonucleotides 6a/b and 7a/b (minigene 1), or
phosphorylated double-stranded oligonucleotides 8a/b and 9a/b
(minigene 2). As a result two pMad5-derived plasmids (pMad5-
1, pMad5-2) were obtained coding for the recombinant proteins

35 depicted in Fig 8. RAd were constructed by transfection of
the Ad5E1-positive cell line 911 (58) with either plasmid
pMad5-1 or pMad5-2 together with plasmid pJM17, which

36

contains the sequence of the Ad5 mutant dl309 (59). 911 cells
were co-transfected with 10 µg of linearized plasmid pMad5-1,
respectively pMad5-2 and 10 µg of plasmid pJM17. The
resulting rAd, which arose through homologous recombination
5   between pMAd5 and pJM17, were 3 times plaque-purified, and
subsequently propagated in 911 cells, purified by double
cesium-chloride density centrifugation and extensively
dialysed. The presence of revertants was routinely checked by
infection of HEP-G2 cells. The viral stocks were stored in
10   aliquots with 10% glycerol at -80°C and titered by plaque
assay using 911 cells.

Transfection of COS-7 cells.

        Transient transfection in COS-7 cells was performed as
15   described elsewhere (60). In short, 100 ng of Plasmids
encoding Ad5E1, HPV16 E7, murine p53, or the influenza-matrix
protein together with 100 ng of a plasmid encoding H-2D^b,
H-2Kb or HLA-A*0201 were transfected by the DEAE-dectran-
chloroquine method into 1x10^4 COS-7 cells. The transfected
20   COS cells were incubated in 100 µl Iscove's modified
Dulbecco's medium containing 8% FCS for 48 h at 37°C, after
which 1500-500 CTL in 25 µl Iscove's modified Dulbecco's
medium containing 50 Cetus Units of recombinant Interleukin-2
(rIL-2, Cetus Corp., Emeryville, CA, USA) were added. After
25   24 h, the supernatant was collected and its tumor necrosis
factor (TNF) content was determined by measuring its
cytotoxic effect on WEHI-164 clone 13 cells as previously
described (60).

30   Infection of MEC with rAd

        B6 MEC were infected with rAd diluted in 1 ml Iscove's
modified Dulbecco's medium containing 0.5% bovine serum
albumine. After 30 minutes at room temperature Iscove's
modified Dulbecco's medium containing 10% FCS was added. The
35   multiplicity of infection (MOI) (for B6 MEC a moi of 50 was
used) was chosen to give at least 80% of infected cell. This
was determined by infection with Ad.RSVß-Gal carrying the

37

Escherichia coli LacZ gene, encoding ß-galactosidase under
control of the promotor from the rous sarcoma virus long
terminal repeat, followed by X-gal staining 48 hours later.

5   Generation of CTL bulk cultures

        5×10^6 spleen cells per well, derived from B6 mice
taken 2 weeks or more after the intra-peritoneal immunization
with 1×10^8 plaque forming units (PFU) of rAd or the
replication-defective Ad5-mutant Ad5 ts 149 were co-cultured
10   for 6 days with 10% irradiated (25GY) IFN-γ (10 units/ml)
treated stimulator cells in 24-wells plates. Next, effector
cells were harvested and dead cells were removed by density
centrifugation on lympholyte M. These cells were used in a
cell-mediated lymphocyte cytotoxicity assay.
15

     Cell-mediated lymphocyte cytotoxicity.

        Experimental procedures to measure cell-mediated
cytotoxicity were performed in an Europium- (Eu^{3+}) release
assay as described elsewhere (56). In short, varying numbers
20   of effector cells were added to 10^3 Eur^{3+}-labeled target
cells in 0.15 ml of culture medium in 96-well U-bottomed
plates. After a 4 hour incubation at 37°C, supernatants were
collected and mixed with Enhancer solution® (Wallac, Turku,
Finland). Measurement of the samples took place in a 1234
25   Delfia® fluorometer (Wallac). The mean percentage specific
lysis of triplicate wells was calculated as follows:
% Specific lysis = [(cpm experimental release - cpm
spontaneous release)/(cpm maximum release - cpm spontaneous
release)] x 100.
30

     Peptides.

        Peptides were generated by solid phase strategies on a
multiple peptide synthesizer (Abimed AMS 422) as described
previously (61).
35

     Tumor cell challenge.

38

C57BL/6 mice were immunized intra-peritoneally with 1 x 10^8 plaque forming units (PFU) or rAd or the replication-defective Ad5-mutant Ad5 ts 149 in 0.25 ml PBS/BSA. Two weeks later the mice were sub-cutaneously challenged with 0.4 x 10^6

5   Ad5ElA + ras cells in 0.25 ml PBS. Tumor volumes were measured with a caliper. Animals were sacrificed when their tumors grew larger than 1000^3 mm to avoid unnecessary suffering.

10   **RESULTS**

Insertion of the coding sequences of several CTL epitopes into pMad5

Vaccination with recombinant viruses encoding intact

15   oncoproteins is intrinsically hazardous, because it can lead to transformation of recombinant virus-infected cells. Therefore, we set out to assemble two minigenes encoding several different CTL epitopes, that were cloned behind the major-late promotor of the vector pMad5. Since we set out to

20   study whether rAd expressing several CTL epitopes in a string-of-bead fashion can be used for vaccination purposes the CTL epitopes used for the construction of the minigene were selected on basis of the availability of CTL clones recognizing the CTL epitopes and/or tumor cells expressing

25   the CTL epitopes. Based upon current knowledge of antigen processing and presentation the CTL epitopes were separated from each other by a spacer of three alanines. The incorporation of the proteolytic cleavage site of three alanines ensures that the encoded CTL eptipes are properly

30   processed (62). The availability of CTL clones recognizing the minigene-encoded CTL epitope is important in order to determine whether the minigene is translated and whether the encoded CTL epitopes are presented in the context of the proper MHC class I-molecule. Likewise, the available murine

35   tumor-models can be used as read-out in order to determine whether the constructed rAd are able, upon vaccination, to

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induce protective respectively therapeutic CTL mediated anti-
cancer immunity.

Based upon these considerations we generated two
recombinant adenoviruses encoding two synthetic minigenes
5   (Fig 8). The synthetic minigenes encoding the CTL epitopes
depicted in figure 8 were cloned into plasmid pMad5 as
described in the material and methods section. All CTL
epitopes encoded by pMad5-1 and two of the four CTL epitopes
encoded by pMad-2 were shown to be processed and presented to
10 tumor-specific CTL as is shown in transient transfection
experiments (Fig 9 and Fig 10). Processing and presentation
of the HPV16 -derived HLA-A2-restricted CTL epitopes
incorporated in pMad5-2 could not be tested, due to the fact
that no CTL clones specific for these peptides are currently
15 available. Nonetheless, our data indicate that these peptides
are expressed and most likely processed, since the last
(Ad5E1B-derived) CTL epitope in the construct is translated,
processed and presented to Ad5E1B-specific CTL. We,
therefore, conclude that all CTL epitopes encoded by pMad5-1
20 and pMad5-2 are translated, processed and presented to CTL
clones.

Since introduction of the minigenes into cells leads to
presentation of the desired CTL epitopes in the context of
the appropriate MHC-restriction molecules, the plasmids
25 pMad5-1 and pMad5-2 harbouring minigene 1 or 2 have been used
to generate replication-defective rAd.

The CTL epitopes encoded by the constructed rAd are processed
and presented to tumor-specific CTL.

30   In order to analyse whether the generated rAd are able,
upon infection, to activate tumor-specific CTL clones, B6 MEC
have been infected with the constructed rAd. These rAd-
infected MEC were used as stimulator cells in a T cell
activation assay, using TNF-prouction as read-out. For
35 reasons of convenience, we focussed in these experiments on
the H-2^b-encoded, virus-derived CTL epitopes. Upon infection
with the rAd encoding minigene 1 (rAd-1), both the Ad5E1A-,

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HPV16 E7-, and the Ad5E1B-derived CTL epitopes are presented
to the appropriate CTL, since these CTL were activated when
incubated with B6 MEC infected with this virus, but not when
incubated with B6MEC infected with a control rAd (Fig 11). By
5   infection of MEC derived from p53 knock-out mice we were able
to show that also the p53-derived CTL epitope was efficiently
processed and presented to p53-specific CTL (data not shown).
Likewise, the rAd encoding minigene 2 (rAd-2) is able to
deliver the Ad5E1B-derived CTL epitope, since infection of B5
10 MEC with this virus leads to activation of Ad5E1B-specific
CTL (Fig 11). Thus, the constructed rAd are able to deliver
all pre-selected CTL epitopes to tumor-specific CTL.

5.   Vaccination of B6 mice with rAd induces tumor-reactive
15   CTL activity.

        Since the rAd are able to deliver all three H-2-
restricted viral CTL epitopes, we have analysed whether
vaccination with these viruses induce CTL activity against
these CTL epitopes. Indeed, bulk CTL cultures derived from B6
20 mice immunized with the rAd-1 display high CTL activity
against the Ad5E1A-. HPVI6 E7-, and the Ad5E1B-encoded CTL
epitopes (Fig. 12 and Fig. 13). Moreover, these CTL bulk
cultures also lyse tumor cells harbouring the relevant CTL
epitopes, showing that the induced CTL display a strong anti-
25 tumor activity. Similarly, vaccination of B6 mice with rAd-2
induced Ad5E1B-specific CTL activity that cross-reacted on
Ad5E1B-expressing tumor cells (Fig. 12). Taken together, these
data show that rAd harbouring synthetic minigenes encoding
several CTL epitopes in a string-bead fashion are able to
30 induce, upon vaccination, strong tumor-specific CTL responses
against the CTL epitopes of choice.

Immunization with rAd-1 induces protective immunity against a
challenge with Ad5E1A + ras transformed tumor cells.
35 The data described above show that immunization wtih
rAd-1 or rAd-2 induce strong tumor-reactive CTL activity
against all tested CTL epitopes. To test whether mice.

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vaccinated with rAd are also protected against a lethal
challenge with tumor cells, we challenged these mice with
tumor cells transformed by the Ad5E1A-region and an activated
ras oncogene (53). These tumor cells only express the Ad5E1A-
5   encoded CTL epitope, and it is therefore anticipated that
rAd-1 only, but not rAd-2, is able to induce protective
immunity against this tumor upon vaccination. Indeed, mice
immunized with rAd-1, but not mice immunized with rAd-2 or
PBS/BSA only, were protected against the outgrowth of Ad5E1A +
10 ras expressing tumor cells (Fig. 14). Moreover, the protection
induced by vaccination with rAd-1 is better than that obtained
by vaccination with irradiated tumor cells, showing that
vaccination with rAd is superior compared to other vaccination
regimes. Thus, vaccination with rAd, harbouring several
15 CTL epitopes, linked with a proteolytic cleavage site, is a
powerful way to induce protective immunity directed against
pre-selected T cell epitopes of choice.

Comments

20   This example shows that rAd encoding defined CTL epitopes
in a string-of-bead fashion, in which the CTL epitopes are
linked to each other by sequences that ensure efficient
processing and presentation of the CTL epitopes are very
potent in inducing protective CTL responses against tumors.
25 All CTL epitopes encoded by the rAd were processed and
presented to tumor- and virus-specific CTL, illustrating that
multiple CTL epitopes can be delivered to the host by a single
vaccination, leading to strong and protective CTL responses.
rAd are easy to manufacture, and do not cause side-effects
30 when used for vaccination, in contrast to other carriers as
vaccinia. Therefore, this method of vaccination is very
effective and safe and is currently being used to deliver
other CTL epitopes described in this invention.

42

Example 5.

Along the same way as described in Example 4, a vaccine
will be prepared, in which the CTL epitopes are incorportated
5   described in Tables X - XX. The vaccine is prepared with the
following characteristics:
a.   the vaccine contains several T cell epitopes linked to
each other by a spacer
b.   the spacers contain proteolytic cleavage sites
10 c.   the T cell epitope containing construct is delivered by a
recombinant adenovirus or is incorporated into the vaccine
types described by point iii - vii on pages 2 and 3.

A vaccine for melanoma is prepared harbouring peptides
15 mentioned in Table XI, a vaccine for colon carcinoma is
prepared harbouzing peptides mentioned in Tables XII and XX, a
vaccine for cervical carcinoma is prepared harbouring peptides
mentioned in Tables XIII - XVI, a vaccine for HIV is prepared
harbouring the peptides mentioned in Table XIX. When
20 appropriate, peptide T cell epitopes other than the ones
listed in Tables X - XX are incorporated into these multi-
epitope vaccines.
The T cell epitopes present in these vaccines are linked
to each other by the following proteolytic cleavage sites (or
25 part of these proteolytic cleavage sites):

AAA as described in (62)

QGW*FEG, WFE*GLF, FEG*LFN, FTT*LIS, TTL*IST, TLI*STI, FNK*SPW,
30 EGL*FNK, TTL*IST, TLI*STI, FNR*SPW as described in (63)

VSG*LEQ, SII*NFE, INF*EKL, LTE*WTS, IIN*FEK, GLE*QLE, EQL*ESI,

NFE*KLT, QLE*SII, EKL*TEW, VVR*FDK, STR*TQI, TQI*NKV,

35

KVV*RFD, VVR*FDK, VRF*DKL, RFD*KLP, DKL*PGF, FGD*SIE,

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VSG*LEQ, QLE*KVV, FDK*LTE, KLT*EWT as described in (64, 65)

LMY*DMY, SEK*RVV, KRV*WMS, DMY*PHF, TNL*GPS, LMY*DMY, as described in (66)

5

LYE*NKP as described in (67)

VNQ*HLC, SHL*VEA, LVE*ALY, EAL*YLV, LYL*VCG as described in (68)

10

VNQ*HLC, QHL*CGS, LVE*ALY, EAL*YLV, ALY*LVC, LYL*VCG,

YLV*CGE, LVC*GER, RGF*FYT, GFF*YTP, FFY*TPK, FYT*PKA,

15   YTP*KA, TPK*A as described in (69)

whereby * represent the site after which the proteasome complex cleaves.

20   RAd vaccines carrying multi-epitope constructs as described above are applied in the appropriate clinical setting as follows:

Dose:
25   between 10^5 and 10^{11} pfu
diluent: isotonic solution; 100 - 1000 µl

Administration:
One to three times, at two-to four-week intervals

30

Possible sites:
Subcutaneous, intra-cutaneous, intra-peritoneal, intramuscular

Clinical evaluations:
35   Inhibition of tumor-growth, regression of existing tumors/metastases

44

Immunological evaluation:
Measurement of T cell responses against relevant and control
peptide T cell epitopes before and after vaccination.

45

<u>Legends to Figures Example 4</u>

Fig. 8. Minigenes encoding several CTL epitopes, linked by
a spacer of three alanines.

The first minigene (rAd-1) encodes an $Ad5E1A_{234-243}$-

5   encoded, $H-2D^b$-restricted CTL epitope (55), an $HPV16E7_{49-59}$-
encoded, $H-2D^b$-restricted CTL epitope (70), a $p53_{158-166}$-
encoded, $H-2K^b$-restricted CTL epitope (unpublished results), an
$Ad5E1B_{192-200}$-encoded, $H-2D^b$-restricted CTL epitope (56), and a
Myc-Tag.

10   The second minigene (rAd-2) encodes an $HPV16\ E7_{86-93}$-
encoded, HLA-A*0201-restricted CTL epitope (71), an Flu-
$matrix_{58-66}$, HLAA*0201-restricted CTL epitope (72), An HPV16
$E7_{11-20}$-encoded, HLAA*0201-restricted CTL epitope (71), an
$Ad5E1B_{192-200}$-encoded, $H-2D^b$restricted CTL epitope (56), and a

15   Myc-Tag.

Fig. 9. Minigene I-encoded CTL epitopes are presented to
tumor-specific CTL clones. pMad5-1 was transfected, together
with a plasmid encoding the appropriate restriction element,

20   into COS-7 cells. After 48 hours, the transfected COS-7 cells
were tested for the expression of the CTL epitopes in their
ability to cause TNF-release by the relevant CTL. The presence
of TNF in the culture supernatant was measured by the
cytotoxic effect on WEHI-164 clone 13 cells.

25

All relevant CTL were activated by COS-7 cells
transfected with a plasmid encoding minigene 1 (but not an
irrelevant control plasmid) together with a plasmid encoding
the appropriate restriction molecule. Thus, minigene 1 is

30   translated into protein and the encoded CTL epitopes are
processed and presented in the context of the appropriate MHC-
molecule to tumor-specific CTL.

Fig. 10. The Flu-derived and Ad5E1B-derived CTL epitopes

35   are presented to Flu-, respectively, Ad5E1B-specific CTL by
minigene 2. pMad5-2 was transfected, together with a plasmid
encoding the appropriate restriction element, into COS-7

46

cells. After 48 hours, the transfected COS-7 cells were tested
for the expression of the CTL epitopes in their ability to
cause TNF-release by the relevant CTL. The presence of TNF in
the culture supernatant was measured by the cytotoxic effect
5   on WEHI-164 clone 13 cells. Relevant CTL were activated by
COS-7 cells transfected with this plasmid (but not an
irrelevant control plasmid) and a plasmid encoding the
appropriate restriction molecule. Tbus, minigene 2 is
translated into protein and encoded CTL epitopes are processed
10   and presented in the context of the appropriate MHC-molecule
to specific CTL.

Fig. 11. CTL epitopes encoded by rAdV are processed, and
presented to tumorspecific CTL. B6 MEC were left uninfected,
15   or were infected with rAd-1 harbouring minigene 1, rAd-2,
harbouring minigene 2 or the galactosidase gene (RAdV-LAC-Z)
at an multiplicity of infection of 50. Two days later these
cells were used in a TNF-production assay as described above.
B6 MEC infected with the rAd-1 harbouring Ad5E1A-, HPV16 E7-
20   and Ad5E1B-derived H-2D^b-restricted CTL epitopes are able to
activate CTL clones. specific for these CTL epitopes, whereas
B6 MEC infected with the rAd-2 harbouring an Ad5E1B-derived
CTL only activate Ad5E1B-specific CTL. The CTL are not
activated upon incubation with uninfected MEC or MEC infected
25   with a control rAd.

Fig. 12. Vaccination with rAdV leads to induction of
tumor-reactive CTL activity against the Ad5E1-encoded CTL
epitopes. B6 mice were left non-immunized, were immunized with
30   rAd-1, harbouring minigene 1, or were immunized with rAd-2,
harbouring minigene 2. Two weeks later the spleens of these
animals were taken and restimulated with Ad5E1-transformed
tumor cells in order to propagate Ad5E1A- and Ad5E1B-specific
CTL. Lytic activity of bulk CTL cultures was tested 6 days
35   later on Ad5E1 MEC, B6 MEC loaded with the the Sendai-virus
encoded control CTL epitope FAPGNYPAL, or the Ad5E1A-encoded
CTL epitope SGPSNTPPE1, or the Ad5E1B-encoded CTL epitope

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VNIRNCCYI, or the HPV16 E7-encoded CTL epitope RAHYNIVTF. Mice
immunized with rAd-1 recognize the Ad5E1A- and Ad5E1B-encoded
CTL epitopes as well as-tumor cells endogenously presenting
the Ad5E1A- and the Ad5E1B-epitope. Mice immunized with rAd-2
5   recognize the Ad5E1B-epitope as well as tumor cells
endogenously presenting the Ad5E1B-encoded CTL epitope,
whereas non-immunized mice do not display reactivity against
the target cells. % specific lysis at different effector to
target cell ratio's is shown.
10

Fig. 13. Vaccination with rAdV leads to the induction
tumor-reactive CTL activity directed against the HPV16 E7, H-
$2D^b$-restricted CTL epitope. B6 mice were left non-immunized,
were immunized with rAd-1, harbouring minigene 1, or were
15   immunized with rAd-2, harbouring minigene 2. Two weeks later
the spleens of these animals were taken and restimulated with
HPV16-transformed tumor cells in order to propagate H-2Db,
HPV16 E7-specific CTL. Lytic activity of bulk CTL cultures was
tested 6 days later on HPV16 MEC, B6 MEC loaded with the the
20   Sendai-virus encoded control CTL epitope FAPGNYPAL, or the
Ad5E1A-encoded CTL epitope SGPSNTPPE1, or the Ad5E1B-encoded
CTL epitope VNIRNCCYI, or the HPVI6 E7-encoded CTL epitope
RAHYNIVTF. Mice immunized with rAd-1 recognize the HPV16 E7-
encoded CTL epitopes as well as tumor cells endogenously
25   presenting the HPV16 E7-epitope. Non-immunized mice and mice
immunized with rAd-2 do not display reactivity against HPV16
E7-peptide positive target cells. % specific lysis at
different effector to target cell ratio's is shown.

30   Fig. 14. Vaccination with rAd-1 induces protective
immunity against a lethal challenge with Ad5E1A-expressing
tumor cells. B6 mice were immunized intraperitoneally with
rAd-1, rAd-2, the Ad5-mutant (Ad5E1A-positive) Ad5ts149, sub-
cutaneously with 10 xO1 irradiated Ad5E1A + ras transformed
35   tumor cells, or were injected with PBS/BSA only. Two weeks
later the mice received a subcutaneous challenge of 0.4 x 10^6
Ad5E1A + ras cells. Mice immunized with rAd-1 and Ad5ts149 are

protected against the outgrowth of Ad5E1A + ras cells, showing
that immunization with rAd induces protective immunity against
tumors.

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§5 References

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TABLE 1   HPV 16 E6 and E7 peptides tested for binding in competition assay at different temperatures for different incubation times.

Sequence[a]	HPV16	IC$_{50}$[b] (nM)	Presence[c] of motif	4°C 3 hr IC$_{50}$[d] (μM)	4°C 24 hr IC$_{50}$ (μM)	26°C 3 hr IC$_{50}$ (μM)	26°C 24 hr IC$_{50}$ (μM)
HLA-A*0201							
TLGIVCPI	E7	7	+	3.9	0.7	6.5	3.1
LLMGTLGIV	E7	8 / 208	-	20	5	10	6.9
YMLDLQPETT	E7	46 / 33	-	2.9	0.7	11	7.3
TLGIVCPIC	E7	153	-	>25	15.7	29	10
KLPQLCTEL	E6	328 / 5556	+	>25	8.3	36	34.5
AMFQDPQER	E6	1818 / >25000	-	>25	17.5	>50	>50
LQTTIHDII	E6	3157 / >25000	-	>25	20.3	>50	>50
FLPSDCFPSV			+	0.8	0.4	1	1.1
HLA-A*0301							
IVYRDGNPY	E6	10 / 660	+	2.1	0.8	2.5	2.5
MLDKKQRFH	E6	68	+	1.9	1.7	9	17
AMFQDPQER	E6	290	-	7.6	3.3	12.5	15
TTLEQQYNK	E6	384	-	16.5	4.2	41	38
IVCPICSQK	E7	1111	+	4.1	3.9	24	24
AHSAARSSR	E6	4285 / 6600	-	4.3	4	6	7
QQLLRREVY	E6	5000 / >6600	-	17	>25	>50	>50
KVFPCALINK		/ 660	+	0.5	0.7	3	4

ᵃ amino-acid sequence of the HPV peptides

ᵇ binding capacity (IC$_{50}$) to the given HLA class I molecule as tested in the molecular binding assay [28]. For some peptides two IC$_{50}$ values are given: HLA-A*0201, the value at the right of the backslash was reported in a later publication [29]; HLA-A*0301, the value at the right side was determined using a molecular binding assay which employs the same FL-labeled reference peptide as used in the cellular binding assay [Drijfhout, manuscript in preparation].

ᶜ the presence of the HLA-A*0201 or HLA-A*0301 binding motif in the peptide

ᵈ binding capacity of the peptides is shown as the concentration of peptide needed to inhibit binding of the FL-labeled peptide to 50% (IC$_{50}$ in μM)

ᵉ the non-labeled reference peptides, the 'ash means that their IC$_{50}$ in the molecular binding assay is not known.

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TABLE II   Binding capacity of known processed and presented peptides

Sequence[a]	IC$_{50}$[b] (nM)	IC$_{50}$[c] (µM)	Origin	Reference
HLA-A*0201				
FLPSDFFPSV	2.8	0.4	hepatitis B nucleocapsid	30
GILGFVFTL	6	0.4	influenza A matrix	31
ILKEPVHGV	242	1.7	HIV-1 RT	32
SLYNTVATL	.	1.3	HIV-1 gag	33
YHNGTMSQV	.	1.7	tyrosinase	34
HLA-A*0301				
QVPLRPHTYK	11	0.5	HIV-1 nef	35
KLFNIHVTY	.	15	unknown	36
KLHKQRAKS	.	12	unknown	36

[a] amino-acid sequence of the peptides

[b] binding capacity (IC$_{50}$ in nM) to the given HLA class I molecule as tested in the molecular binding assay [29]

[c] binding capacity of the peptides in the present study is shown as the concentration of peptide needed to inhibit binding of the FL-labeled peptide to 50% (IC$_{50}$ in µM)

TABLE III   Binding of conserved HIV-1 pol sequences compliant with the HLA-A'0301 binding motif

Sequence[a]	Position[b]	IC_{50} $(\mu M)^{c}$	
PISPIETVPVK	160 - 170	>100	
PIETVPVKLK	163 - 172	>100	
PIETVPVK	163 - 170	>100	
PLTEEKIK	184 - 191	>100	
AIKKKDSTK	221 - 229	1.0 - 3.0	*
GIPHPAGLK	252 - 260	0.3 - 0.5	*
SVTVLDVGDAY	264 - 274	>100	
TVLDVGDAY	266 - 274	>100	
VIDVGDAY	267 - 274	>100	
NVLPQGWK	306 - 313	30.0 - 40.0	
WHGYELHPDK	388 - 397	14.0 - 20.0	
ELELAENR	459 - 466	>100	
ELAENREILK	461 - 470	14.0 - 20.0	
QLDCTHLEGK	781 - 790	8.5 - 10.0	
AVHVASGY	795 - 802	21.5 - 25.0	
QVRDQAEHLK	883 - 892	2.9 - 3.0	*
AVFIHNFKR	898 - 906	0.3 - 0.5	*
GIGGYSAGER	909 - 918	6.5 - 10.0	
KIQNFRVYY	938 - 946	1.8 - 2.5	*
KIQNFRVY	938 - 945	70.0 - 90.0	

[a] The amino-acid sequence of conserved peptides derived from HIV-1

[b] position of first and last amino-acid in HIV-1 polymerase derived from strain JR-CSF

[c] Peptides were tested in the competition assay at 4°C with an incubation time of 24 hours. The binding capacity of the peptides is shown as the range of the concentration of peptide needed to inhibit binding of the FL-labeled peptide to 50% (IC_{50} in μM). The peptides that are marked with a asterisk (*) are considered to be potential CTL epitopes.

Table **IV**   Comparison of the immunogenicity of Hepatitis B virus (HBV) or

Human Papilloma virus type 16 (HPV16) derived peptides to the

dissociation rate.

Peptide	amino-acid position	Sequence	Affinity IC50 (nM)	IC50 (µM)	Immuno-genicity	Stability (DT50%)
HBV Pol [a]	635- 643	GLYSSTVPV	33	4.5 [b]	+ [c]	> 4 hr [d]
HBV Pol	1076-1084	HLYSHPIIL	38	8.0	+	> 4 hr
HBV Pol	1344-1352	WILRGTSFV	278	11.0	-	1 hr
HBV Pol	996-1004	NLSWLSLDV	385	6.0	+	3 hr
HBV Pol	992-1000	LLSSNLSWL	1087	19.5	-	1 hr
HBV Pol	985- 993	NLQSLTNLL	2000	22.0	-	NS
HBV Pol	43- 51	HLLVGSSGL	2778	24.0	-	< 1 hr
HBV Pol	28- 36	LLDDEAGPL	>25000	69.0	-	NS
HBV Pol	594- 602	PLEEELPRL	>25000	>100	-	NS
HPV16 E7	86- 93	TLGIVCPI	7	0.7	+	> 4 hr
HPV16 E7	11- 20	YMLDLQPETT	46	0.7	+	> 4 hr
HPV16 E6	52- 60	FAFRDLCIV	130	9.0	-	2 hr
HPV16 E7	7- 15	TLHEYMLDL	188	5.0	-	2 hr
HPV16 E7	82- 90	LLMGTLGIV	208	5.0	+	> 4 hr
HPV16 E6	18- 26	KLPQLCTEL	328	8.5	-	2 hr
HPV16 E6	7- 15	AMFQDPQER	1818	17.5	-	NS
HPV16 E6	26- 34	LQTTIHDII	3157	20.5	-	NS

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Table IV

a   Peptide origin, position of first and last amino-acid and amino-acid
     sequence and binding affinity as described previously (10,24).

b   Affinity was measured as described recently (17). IC_{50} represents the
     amount of peptide required for 50% inhibition of binding of the
     fluorescein-labeled reference peptide to HLA-A*0201

c   Immunogenicity of the peptide was determined by injection of peptide
     doses of 10- to 100-fold in excess of what is required to elicit optimal
     CTL responses emulsified in IFA together with an equimolar amount of I-
     A^{b} T-helper epitope (10.11): - non-immunogenic, + immunogenic

d   The time required for 50% of the molecules to decay (DT50%) is given
     starting from t = 2 hours at 37°C. NS = non stable: < 10% of HLA
     molecules were detectable after a 2 hour incubation at 37°C.

Table V   Comparison of peptide binding affinity, dissociation rate and
immunogenicity of HBV and HPV16 derived peptides.

Dissociation rate DT50%

Peptide binding affinity	≥ 3 hours	< 3 hours	
high	3	0	immunogenic
	0	0	non-immunogenic
intermediate	3	0	immunogenic
	0	4	non-immunogenic
low	0	0	immunogenic
	0	7	non-immunogenic

Table VI   The stability of HLA-A*0201 complexed with known CTL epitopes.

Peptide	First aa position	Sequence	Affinity IC50 (µM)	Stability (DT50%)		Immunogenicity
			a	b	c	d
HCV1 core	131	ADLMGYIPLV	50.0	> 4	hr	RC
HCV1 core	178	LLALLSCLTV	7.5	> 4	hr	RC
HCV1 NS3	1406	KLVALGINAV	5.0	4	hr	RC
HCV1 NS4	1789	SLMAFTAAV	1.5	> 4	hr	RC
HBV surface	335	WLSLLVPFV	1.0	> 4	hr	RC
HBV surface	348	GLSPTVWLSV	2.0	> 4	hr	RC
EBV LMP2	426	CLGGLLTMV	2.5	4	hr	PR1
HTLV1 tax	11	LLFGYPVYV	0.8	> 4	hr	RC
HPV11 E7	4	RLVTLKDIV	52.0	2	hr	PR2
INF B NP	85	KLGEFYNQMM	5.5	> 4	hr	CTL
INF A Matrix	58	GILGFVFTL	0.6	> 4	hr	CTL
HIV-1 Gag	76	SLYNTVATL	1.5	> 4	hr	CTL
HIV-1 Pol	267	VLDVGDAYFSV	7.0	NS		PR3
HIV-1 Pol	468	ILKEPVHGV	8.0	> 4	hr	CTL
pmel17/gp100 [1]		YLEPGPVTA	8.5	4	hr	CTL
pmel17/gp100 [2]		LLDGTATLRL	5.5	4	hr	CTL
tyrosinase	369	YMNGTMSQV	4.5	> 4	hr	CTL

Table VI

a   Peptide origin, position of first amino-acid and amino-acid sequence of the different HLA-A*0201 restricted CTL epitopes are given (20,25-36).

b   Binding affinity was measured as described recently (17). IC_{50} represents the amount of peptide required for 50% inhibition of binding of the fluorescein-labeled reference peptide to HLA-A*0201

c   The time required for 50% of the molecules to decay (DT50%) is given starting from t = 2 hours at 37°C. NS = non stable, < 10% of HLA molecules were detectable after a 2 hour incubation at 37°C.

d   RC: recall experiment wherein CTL already primed by viral infection of the patient in vivo were boosted in vitro with peptide to detect the precise epitope. All authors used similar protocols. CTL: peptides were used to identify the epitopes recognized by CTL which were obtained from patients. PR1: CTL were primed in vitro with an autologous EBV transformed B-cell line and then cloned, peptides were used to map the epitope recognized. PR2: CTL were induced in vitro using repeated stimulation with recombinant vaccinia virus-HPV11 E7 infected B-cells. PR3: CTL were induced in vitro using repetitive stimulation with peptide pulsed antigen presenting cells.

Table VII   Statistical analysis of the dissociation rate (DT50%) or binding

affinity versus immunogenicity of HLA-A*0201 binding peptides.

	immunogenic	non-immunogenic
DT50% ≥ 3 hr	21	0
DT50% < 3 hr	2	11
		p=0.0000003 [a]
high affinity	11	0
intermediate affinity	10	4
low affinity	2	7
		p=0.0005 [b]

[a]) Fisher's 2-tailed exact test for 2 by 2 tables.

[b])The relation between binding affinity and immunogenicity was
determined by comparison of the high-affinity binding peptides with
the low-affinity binding peptides. using a Fisher's 2-tailed exact
test for 2 by 2 tables.

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Table VII   Statistical analysis of dissociation rate (DT50%) *or* binding affinity

versus immunogenicity of peptides binding with intermediate- or

low affinity to HLA-A*0201.

	immunogenic	non-immunogenic
DT50% $\geq$ 3 hr	10	0
DT50% < 3 hr	2	11
		p=0.00007 *
intermediate affinity	10	4
low affinity	2	7
		p=0.04 *

* Fisher's 2-tailed exact test for 2 by 2 tables.

Table VIII Immunogenicity of HIV-1 derived peptides with known dissociation

rate tested in HLA-A*0201/Kb transgenic mice

Sequence+ origin	Affinity IC_{50} (μM)	Stability DT50%		LU30%/10^6 cells	CTL response
	a	b	c	d	e
FLPSDDFPSV HBVcore-18	0.4	> 4	hr	53 (25-71)	3/3
TLGIVCPI HPV16E7-86	0.7	> 4	hr	183 (70-400)	3/3
VLDVGDAYFSV HIV-1pol-267	7.0	NS		< 2	0/7
YMDDLYVGSDL HIV-1pol-343	8.0	1	hr	< 2	0/3
LLWKGEGAV HIV-1pol-576	6.0	> 4	hr	84 (67-100)	2/3
ILKEPVHGV HIV-1pol-468	8.0	> 4	hr	56 (17-100)	5/6

Table VIII

a   Peptide amino-acid sequence, protein and position of the first amino-acid
     of the different HLA-A*0201 binding potential CTL epitopes are given.

b   Average binding affinity was measured as described recently (17). IC_{50}
     represents the amount of peptide required for 50% inhibition of binding of
     the fluorescein-labeled reference peptide to HLA-A*0201

c   The time required for 50% of the molecules to decay (DT50%) is given
     starting from t = 2 hours at 37°C. NS = non stable: < 10% of HLA
     molecules were detectable after a 2 hour incubation at 37°C.

d   Average of all mice and range of observed responses.

e   Number of mice which mounted a peptide-specific CTL response per total
     mice vaccinated.

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Table IX: Relation between peptide binding affinity, stability of the MHC peptide complex and immunogenicity of the peptide.

Peptide	source[1]	T2-assay F.I.[2]	B cell assay IC50[3]	Stability assay DT50% in h.[4]	Immuno-genicity[6]
Melan-A/MART-1-derived peptides					
EAAGIGILTV	aa26-35	0.58	15	NS[5]	-
AAGIGILTV	aa 27-35	0.03	80	>4	+
GILTVILGV	aa 31-39	0.95	6	>4	+
ALMDKSLHV	aa 56-64	0.96	7	>4	+
Positive control peptides					
GILGFVFTL	Flu-M1	1.35	0.6	>4	+
YLEPGPVTA	pmel17/gp100	0.93	8.5	4	+
LLDGTATLRL	pmel17/gp100	0.69	4	4	+
YMDGTMSQV	tyrosinase	0.60	1.2	>4	+

Legend to Table IX:

1) Protein sources from which peptides have been derived are
mentioned. For Melan-A/Mart-1-derived peptides the respective
aa positions are indicated. Positive control peptides have

5   been described elswhere (40, 45-47).

2) Fluorescence index (F.I.) is calculated for binding of
peptides to T2 cells at 25 µg/ml. The T2 binding assay has
been described elswhere (2).

3) Concentration of the peptide that inhibits 50% of maximal

10   binding of reference HBV core peptide. This binding assay
employing MHC class I molecules on intact B cells is
described under Example 1.

4) Relative stability of peptide binding to HLA-A*0201,
calculated as DT50% see Example 2 for experimental

15   procedures).

5) NS = not stable; DT50% not calculated because of absence
of peptide binding after 2 hrs.

6) Immunogenicity of peptide (see text Example 3).

Table X

Preselected peptides having an amino acid sequence derived from human influenza M protein, wherein said amino acid sequence has the ability to bind to human MHC Class I allele HLA-A2.1 and is selected from the group consisting of:

Peptide	Amino acid sequence	location in influenza M protein
1	S L L T E V E T Y V	(residues 2-11 of M protein)
2	S L L T E V E T Y V L	(residues 2-12 of M protein)
3	L L T E V E T Y V	(residues 3-11 of M protein)
4	L L T E V E T Y V L	(residues 3-12 of M protein)
5	V L M E W L K T R P I	(residues 41-51 of M protein)
6	P I L S P L T K G I	(residues 50-59 of M protein)
7	I L S P L T K G I	(residues 51-59 of M protein)
8	I L S P L T K G I L	(residues 51-60 of M protein)
9	G I L G F V F T L	(residues 58-66 of M protein)
10	G I L G F V F T L T V	(residues 58-68 of M protein)
11	I L G F V F T L T V	(residues 59-68 of M protein)
12	R M G A V T T E V	(residues 134-142 of M protein)
13	G L V C A T C E Q I A	(residues 145-155 of M protein)
14	Q M V T T T N P L	(residues 164-172 of M protein)
15	Q M V T T T N P L I	(residues 164-173 of M protein),

The following table presents preselected peptides derived
from human melanoma associated protein tyrosinase capable of
upregulating HLA-A*0201 molecules on T2 cells.

5 <u>Table XI</u>

Peptide No.	Sequence	Residues
	CLLWSFQTSA	008-017
1	LLWSFQTSA	009-017
	RLLVRRNIFDL	116-126
2	YLTLAKHTI	137-145
	TISSDYVIPI	144153
	PAFLPWHRLFL	205-215
3	FLPWHRLFL	207-215
4	FLPWHRLFLL	207-216
5	FLLRWEQEI	214222
6	TLEGFASPL	343-351
7	FASPLTGIADA	347-357
8	SMHNALHIYM	361-370
	HIYMNGTMSQV	367-377
9	YMNGTMSQV	369-377
	PIFLLHHAFV	384393
	WLQRHRPLQEV	400-410
10	PLYRNGDFFI	431-440
11	YIKSYLEQA	463-471
	RIWSWLLGA	473-481
	RIWSWLLGAAM	473-483
12	WLLGAAMVGA	477-486
13	MVGAVLTAL	483-491
	VLTALLAGPV	487-496
	LTALLAGPVSL	488-498
	TALLAGPVSL	489-4
	TALLAGPVSLL	489-499
14	ALLAGPVSL	490-498
15	ALLAGPVSLL	490-499
16	LLAGPVSLL	491-499
	QLPEEKQPLL	506-515
17	LLMEKEDYHSL	514-524

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Table XII.

Preselected peptides having an amino acid sequence derived from
p53, wherein said amino acid sequence has the ability to bind to
human MHC Class I allele HLA-A2.1 and is selected from the group
5   consisting of:

Peptide   Amino acid   location in human p53 protein
          sequence
_____

     1.   LLPENNVLS   (residues 25-33 of human p53)
     2.   RMPEAAPPV   (residues 65-73 of human p53)
     3.   FLHSGTAKSV   (residues 113-122 of human p53)
10   4.   KMFCQLAKT   (residues 132-140 of human p53)
     5.   KQSQHMTEV   (residues 164-172 of human p53)
     6.   HMTEVVRRC   (residues 168-176 of human p53)
     7.   DRNTFRHSVV   (residues 208-217 of human p53)
     8.   LLGRNSFEV   (residues 264-272 of human p53)
15   9.   KMLCQLAKT   (residues 132-140 of human p53)
     10. NMFCQLAKT   (residues 132-140 of human p53)
     11. KLFCQLAKT   (residues 132-140 of human p53)
     12. QMFCQLAKT   (residues 132-140 of human p53)
     13. KMFTQLAKT   (residues 132-140 of human p53)
20   14. KMFYQLAKT   (residues 132-140 of human p53)
     15. KMFCELAKT   (residues 132-140 of human p53)
     16. KMFCQLAKY   (residues 132-140 of human p53)
     17. NLFCQLAKT   (residues 132-140 of human p53)
     18. QQSQHMTEV   (residues 164-172 of human p53)
25   19. HMTEVLRRC   (residues 168-176 of human p53)
     20. HMTEVVRLC   (residues 168-176 of human p53)
     21. HMTEVVRRF   (residues 168-176 of human p53)
     22. HMTEVVRHC   (residues 168-176 of human p53)
     23. DRNAFRHSVV   (residues 208-217 of human p53)
30   24. DRNTFRHSMV   (residues 208-217 of human p53)
     25. LLVRNSFEV   (residues 264-272 of human p53)
     26. LLGRNSFEM   (residues 264-272 of human p53)
     A.   ILTIITLED   human p53 residues 251-259

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B.	MLSPDDIEQ	human p53 residues	44-52
C.	IRVEGNLRV	human p53 residues	195-203
D.	KLMFKTEGP	human p53 residues	382-390
E.	DLWKLLPEN	human p53 residues	21-29
F.	ALPNNTSSS	human p53 residues	307-315
G.	LHSGTAKSV	human p53 residues	114-122
H.	NLRKKGEPH	human p53 residues	288-297
I.	PLSSSVPSQ	human p53 residues	92-100
J.	ELPPGSTKR	human p53 residues	298-306
K.	FLHSGTAKS	human p53 residues	113-121

5 at F., 10 at K.

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Preselected peptides having an amino acid sequence derived from
HPV16/18 protein, wherein said amino acid sequence has the
ability to bind to human MHC Class I allele HLA-A2.1 and is
selected from the group consisting of:

5

Table XIII.
Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A2.1

Peptide No.	Amino acid sequence	protein (region)	SEQ NO
-	AMFQDPQER	E6 (residues 7- 15)	1
1	KLPQLCTEL	E6 (residues 18 - 26)	2
2	QLCTELQTT	E6 (residues 21 - 29)	3
3	LCTELQTTI	E6 (residues 22 - 30)	4
4	ELQTTIHDI	E6 (residues 25 - 33)	5
5	LQTTIHDII	E6 (residues 26 - 34)	6
6	TIHDIILEC	E6 (residues 29 - 37)	7
7	IHDIILECV	E6 (residues 30 - 38)	8
8	CVYCKQQLL	E6 (residues 37 - 45)	9
-	FAFRDLCIV	E6 (residues 52- 60)	10
9	KISEYRHYC	E6 (residues 79 - 87)	11
10	PLCDLLIRC	E6 (residues 102-110)	12
11	TLHEYMLDL	E7 (residues 7 - 15)	13
12	YMLDLQPET	E7 (residues 11 - 19)	14
13	MLDLQPETT	E7 (residues 12 - 20)	15
14	RLCVQSTHV	E7 (residues 66 - 74)	16
15	TLEDLLMGT	E7 (residues 78 - 86)	17
16	LLMGTLGIV	E7 (residues 82 - 90)	18
17	GTLGIVCPI	E7 (residues 85 - 93)	19
18	TLGIVCPIC	E7 (residues 86 - 94)	20

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TABLE XIV

Peptides derived from HPV18 proteins E6 and E7 binding to HLA-A2.1

Peptide No.	Amino acid sequence	protein (region)	SEQ NO
1	KLPDLCTEL	E6 (residues 13 - 21)	21
2	SLQDIEITC	E6 (residues 24 - 32)	22
3	LQDIEITCV	E6 (residues 25 - 33)	23
4	EITCVYCKT	E6 (residues 29 - 37)	24
5	KTVLELTEV	E6 (residues 36 - 44)	25
6	ELTEVFEFA	E6 (residues 40 - 48)	26
7	FAFKDLFVV	E6 (residues 47 - 55)	27
8	DTLEKLTNT	E6 (residues 88 - 96)	28
9	LTNTGLYNL	E6 (residues 93 -101)	29
10	TLQDIVLHL	E7 (residues 7 - 15)	30
11	FQQLFLNTL	E7 (residues 86 - 94)	31
12	QLFLNTLSF	E7 (residues 88 - 96)	32
13	LFLNTLSFV	E7 (residues 89 - 97)	33
14	LSFVCPWCA	E7 (residues 94 -102)	34

TABLE XV

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A1

Amino acid sequence	protein (region)	SEQ NO
YRDGNPYAV	E6 (residues 61- 69)	35
WTGRCMSCC	E6 (residues 139-147)	36
MSCCRSSRT	E6 (residues 144-152)	37
TTDLYCYEQ	E7 (residues 19- 27)	38
EIDGPAGQA	E7 (residues 37- 45)	39
HVDIRTLED	E7 (residues 73- 81)	40

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TABLE XVI

Peptides derived from HPV16 proteins E6 and E7 binding to H̲LA-A3.2

	Amino acid sequence	protein (region)	SEQ NO
	AMFQDPQER	E6 (residues 7- 15)	1
	IILECVYCK	E6 (residues 33- 41)	41
	CVYCKQQLL	E6 (residues 37- 45)	9
10	VYCKQQLLR	E6 (residues 38- 46)	42
	QQLLRREVY	E6 (residues 42- 50)	43
	IVYRDGNPY	E6 (residues 59- 67)	44
	YAVCDKCLK	E6 (residues 67- 75)	45
	AVCDKCLKF	E6 (residues 68- 76)	46
15	VCDKCLKFY	E6 (residues 69- 77)	47
	KFYSKISEY	E6 (residues 75- 83)	48
	KISEYRHYC	E6 (residues 79- 87)	11
	ISEYRHYCY	E6 (residues 80- 88)	49
	RHYCYSLYG	E6 (residues 84- 92)	50
20	SLYGTTLEQ	E6 (residues 89- 97)	51
	TTLEQQYNK	E6 (residues 93-101)	52
	QQYNKPLCD	E6 (residues 97-105)	53
	LIRCINCQK	E6 (residues 107-115)	54
	HLDKKQRFH	E6 (residues 125-133)	55
25	CMSCCRSSR	E6 (residues 143-151)	56
	SCCRSSRTR	E6 (residues 145-153)	57
	CCRSSRTRR	E6 (residues 146-154)	58
	HYNIVTFCC	E7 (residues 51- 59)	59
	YNIVTFCCK	E7 (residues 52- 60)	60
30	CCKCDSTLR	E7 (residues 58- 66)	61
	KCDSTLRLC	E7 (residues 60- 68)	62

TABLE XVII

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A11.2

Amino acid sequence	protein (region)	SEQ NO
AMFQDPQER	E6 (residues 7- 15)	1
IILECVYCK	E6 (residues 33- 41)	41
CVYCKQQLL	E6 (residues 37- 45)	9
VYCKQQLLR	E6 (residues 38- 46)	42
QQLLRREVY	E6 (residues 42- 50)	43
IVYRDGNPY	E6 (residues 59- 67)	44
YAVCDKCLK	E6 (residues 67- 75)	45
AVCDKCLKF	E6 (residues 68- 76)	46
VCDKCLKFY	E6 (residues 69- 77)	47
KISEYRHYC	E6 (residues 79- 87)	11
ISEYRHYCY	E6 (residues 80- 88)	49
LIRCINCQK	E6 (residues 107-115)	54
TGRCMSCCR	E6 (residues 140-148)	63
CMSCCRSSR	E6 (residues 143-151)	56
SCCRSSRTR	E6 (residues 145-153)	57
HYNIVTFCC	E7 (residues 51- 59)	59
YNIVTFCCK	E7 (residues 52- 60)	60
CCKCDSTLR	E7 (residues 58- 66)	61
VCPICSQKP	E7 (residues 90- 98)	64

TABLE XVIII

Peptides derived from HPV16 proteins E6 and E7 binding to HLA-A24

5	Amino acid sequence	protein (region)	SEQ NO
	MHQKRTAMF	E6 (residues 1- 9)	65
	LQTTIHDII	E6 (residues 26- 34)	6
10	VYCKQQLLR	E6 (residues 38- 46)	42
	LLRREVYDF	E6 (residues 44- 52)	66
	VYDFAFRDL	E6 (residues 49- 57)	67
	PYAVCDKCL	E6 (residues 66- 74)	68
	KCLKFYSKI	E6 (residues 72- 80)	69
15	EYRHYCYSL	E6 (residues 82- 90)	70
	HYCYSLYGT	E6 (residues 85- 93)	71
	CYSLYGTTL	E6 (residues 87- 95)	72
	RFHNIRGRW	E6 (residues 131-139)	73
	RAHYNIVTF	E7 (residues 49- 57)	74

Table XIX
Preselected peptides having an amino acid sequence derived from
HIV, wherein said amino acid sequence has the ability to bind to
human MHC Class I allele HLA-A2.1 and is selected from the group
consisting of:

```
1.E M M T A C Q G V
2.L L D T G A D D T V
3.V L D V G D A Y F S V
4.L L W K G E G A V
5.I L K E P V H G V
```

Table XX.

Preselected peptides having an amino acid sequence derived from
CEA, wherein said amino acid sequence has the ability to bind to
human MHC Class I allele HLA-A2.1 and is selected from the group
5   consisting of:

Peptide no.	Amino acid sequence	Residues
A1	Q I I G Y V I G T	044-052
A2	Y L W W V N N Q S L	142-151
A3	V L Y G P D A P T I	199-208
A4	V L Y G P D T P I	555-563
A5	Y L S G A N L N L	571-579

Peptides derived from CEA binding to the HLA-A*0301 molecule

Peptide no.	Amino acid sequence	Residues
B1	H L F G Y S W Y K	027-035
B2	R V D G N R Q I I G Y	038-048
B3	R V Y P E L P K	105-112
B4	R L Q L S N D N R	334-342
B5	E L F I S N I T E K	427-436
B6	L F I S N I T E K	428-436
B7	F I S N I T E K	429-436
B8	T L T L F N V T R	521-529
B9	T L F N V T R N D A R	523-533
B10	N V T R N D A R	526-533
B11	F V S N L A T G R	622-630

Table A the oligonucleoties used in these studies in order to
generate rAdV that encoded CTL epitopes in a string-of-bead
fashion, linked with proteolytic cleavage sites that direct
CTL epitope processing.

1a   CGCGAATTATGAACGCGTC
1b   GTACGACGCGTRCATAATT

2a   GTACGCTACTAGTGAACAGAAGCTGATATCAGAGGAAGACCTAAACTGAT
2b   CTAGATCAGTTTAGG CWMATATCAGCTTCTGTTCACTAGTAGC

3a   CGCGGCAGCTTCCGGTCCTTCTAACACACCTCCTGAGATAGCAGCC
3b   GCTATCTCAGGAGGTGTGTTAGAAGGACCGGAAGCTGC

4a   CTGTAAATATCAGGAATTGTTGCTACATTGCAGCTG
4b   CTAGCAGCTGCAATGTAGCAACAATTCCTGATATTTACAGCTGC

5a   CGCGGCAGCTACACTAGGAATTGTGTGCCCCATCGCAGCC
5b   GCGATGGGGCACACAATTCCTAGTGTAGCTGC

6a   GCTAGAGCCCATTACAATATTGTAACCTTTGCTGCG
6b   GCAAAGGTTACAATATTGTAATGGGCTCTAGCGGCT

7a   GCTGCCATCTACAAGAAGTCACAGCACATGGCTGCAG
7b   ACGCCGACGGTAGATGTTCTTCAGTGTCGTGTACCGA

8a   GCTGGAATCCTAGGTTTCGTCTTTACGCTAGCTGCG
8b   GCTAGAGTAAAGACGAAACCTAGGATTCCAGCGGCT

9a   GCTTATATGTTAGATTTGCAACCAGAGACAACTGCTGCAG
9b   AGCAGTTGTCTCTGGTTGCAAATCTAACATATAAGCCGCA

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CLAIMS

1.   A method for the selection of immunogenic T cell peptide
epitopes present in polypeptide antigens comprising
identification of peptides in the primary sequence of the
antigen having a binding motif and size for binding to a HLA
5   class I molecule, measuring the binding of said identified
peptides to MHC class I molecules, whereby the stability of
the complex of the peptide and the MHC class I molecule is
measured on intact cells carrying said MHC class I molecule at
their surfaces.

10   2.   A method according to claim 1 whereby the intact cells
are B cells.

3.   A method according to claim 2 whereby the B cells are
human B cells.

4.   A method according to any one of the aforegoing claims
15   comprising a further selection step of peptides whereby the
peptides and their flanking sequences in the intact antigen
are screened for compliance with the rules for proteasome
cleavage of natural polypeptides sequences.

5.   A method according to any one of the aforegoing claims
20   whereby the peptides and their flanking sequences in the
intact antigen are screened for compliance with the rules for
peptide transport and/or peptide loading onto HLA class I
molecules.

6.   A method according to any one of the aforegoing claims
25   further comprising a further binding assay for the binding of
identified peptides to MHC class I molecules.

7.   A method according to claim 6 wherein the further binding
assay measures the binding of identified peptides to empty MHC
class I molecules at the surface of an antigen processing
30   defective cell line.

8.   A method according to claim 7 wherein the processing
defective cell line is the T2 cell line.

9.   A method for the production of a vaccine for the
treatment and/or prophylaxis of a disease associated with the

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presence of a polypeptide antigen, comprising selecting T cell peptide epitopes of said polypeptide antigen by a method according to any one of claims 1-8, preparing selected peptide epitopes and mixing said peptide epitopes with a vehicle

5 suitable for administration.

10. A method for producing a vaccine according to claim 9 whereby an adjuvans is added to the vaccine.

11. A method according for producing a vaccne according to claim 9 or 10, whereby the peptide epitope comprises a

10 synthetic peptide.

12. A method according to claim 11 whereby the vaccine comprises a mixture of different synthetic peptides.

13. A method according to claim 11 or 12 whereby the synthetic peptide is loaded onto a dendritic cell.

15 14. A method according to anyone of laims 9-12, whereby the peptide epitopes are present in a string-bead conformation.

15. A method according to claim 14 whereby the strings comprises proteolytic cleavage sites.

16. A method according to claim 9 or 10 whereby a peptide

20 epitope is provided as part of a recombinant protein.

17. A method according to claim 16 whereby the recombinant protein has a string bead conformation whereby the beads are peptide epitopes.

18. A method according to claim 17 whereby the strings

25 comprise proteolytic cleavage sites.

19. A method according to any one of claims 16-18 whereby the recombinant protein is loaded on a dendritic cell.

20. A recombinant protein comprising a peptide epitope selected according to a method of anyone of claims 1-8.

30 21. A recombinant protein according to claim 20 which protein has a string-bead conformation wherein the beads are peptide epitopes selected according to a method of anyone of claims 1-8.

22. A recombinant protein according to claim 21 whereby the

35 strings comprise proteolytic cleavage sites.

23. A recombinant nucleic acid molecule encoding a recombinant protein according to anyone of claims 20-22.

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24. A recombinant nucleic acid according to claim 23 which
is a recombinant Canary pox virus nucleic acid.
25. A recombinant nucleic acid according to claim 23 which
is a recombinant human adenovirus nucleic acid.
5   26. A recombinant Canary pox virus comprising a nucleic acid
according to claim 24 and/or which comprises a recombinant
protein encoded by said nucleic acid.
27. A recombinant human adenovirus comprising a nucleic acid
according to claim 25 and/or which comprises a recombinant
10   protein encoded by said nucleic acid.
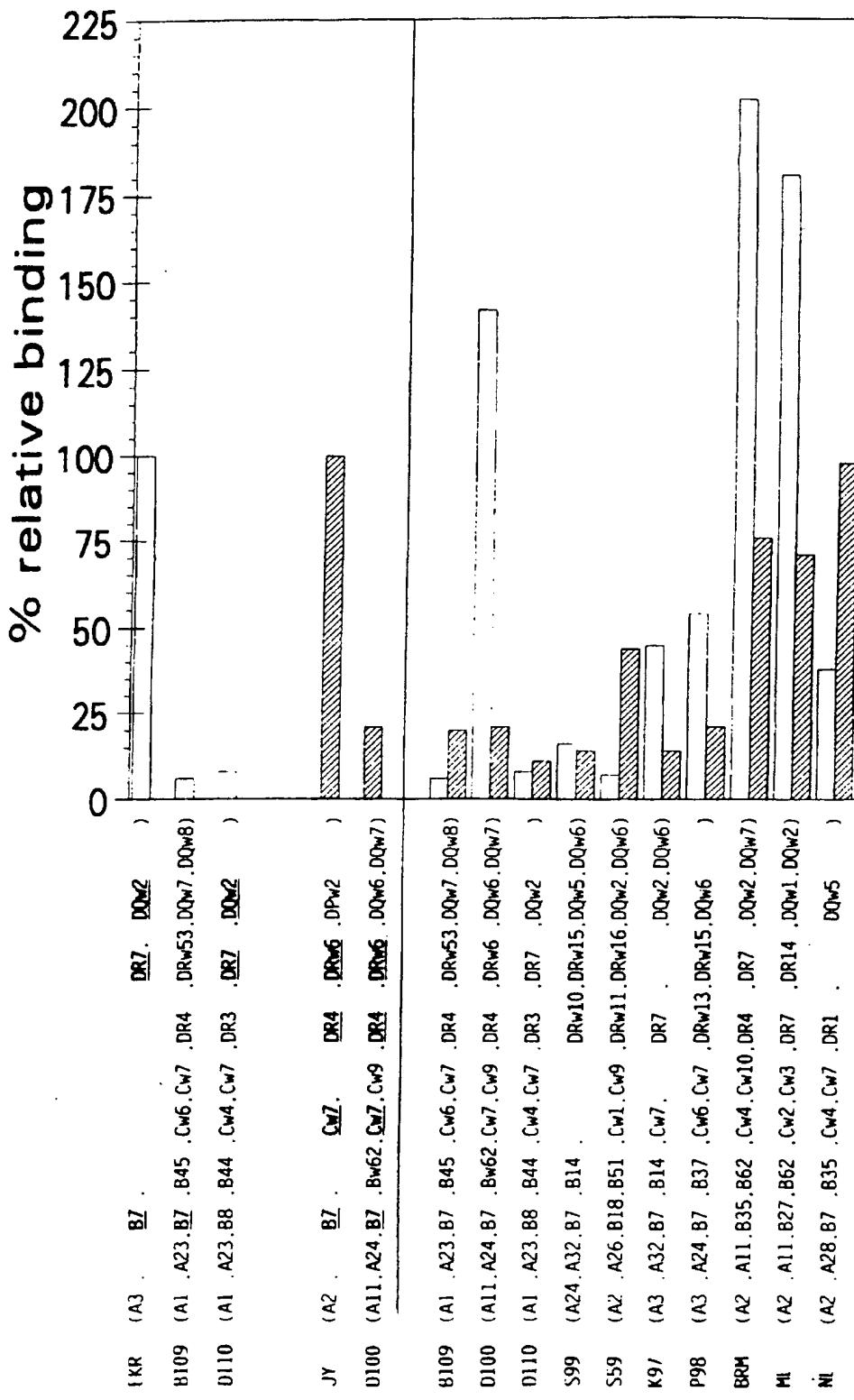
FIG. 1
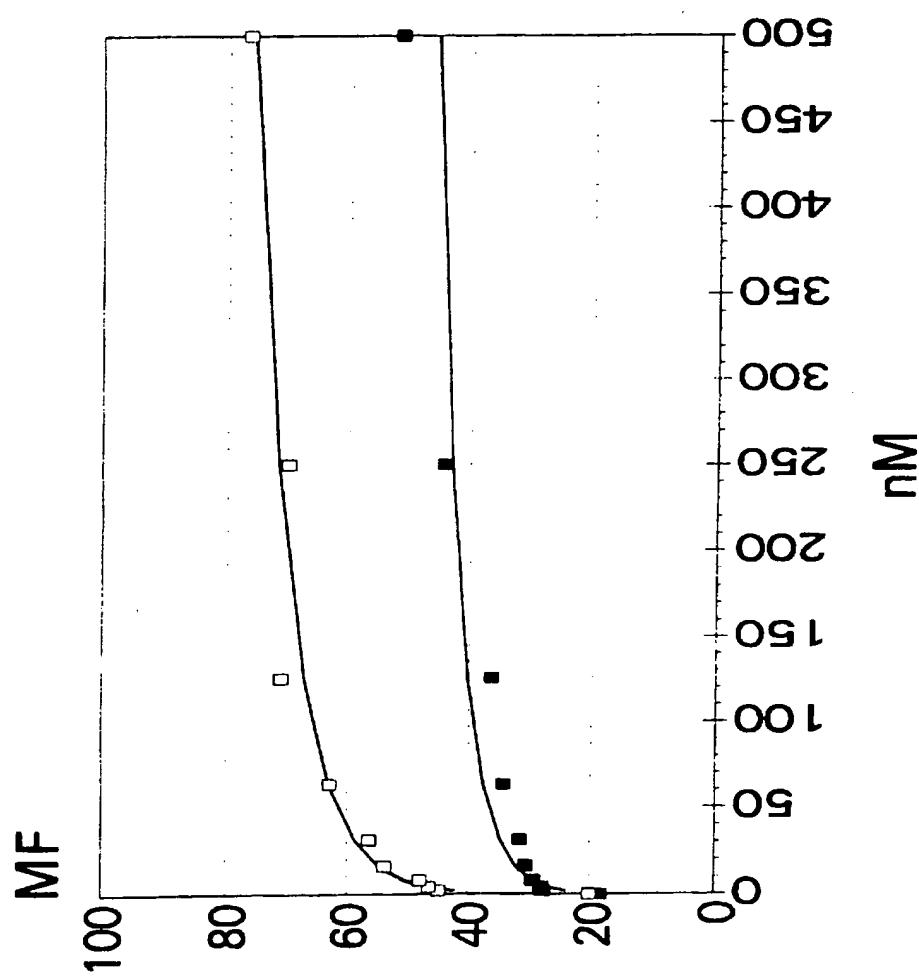
FIG. 2
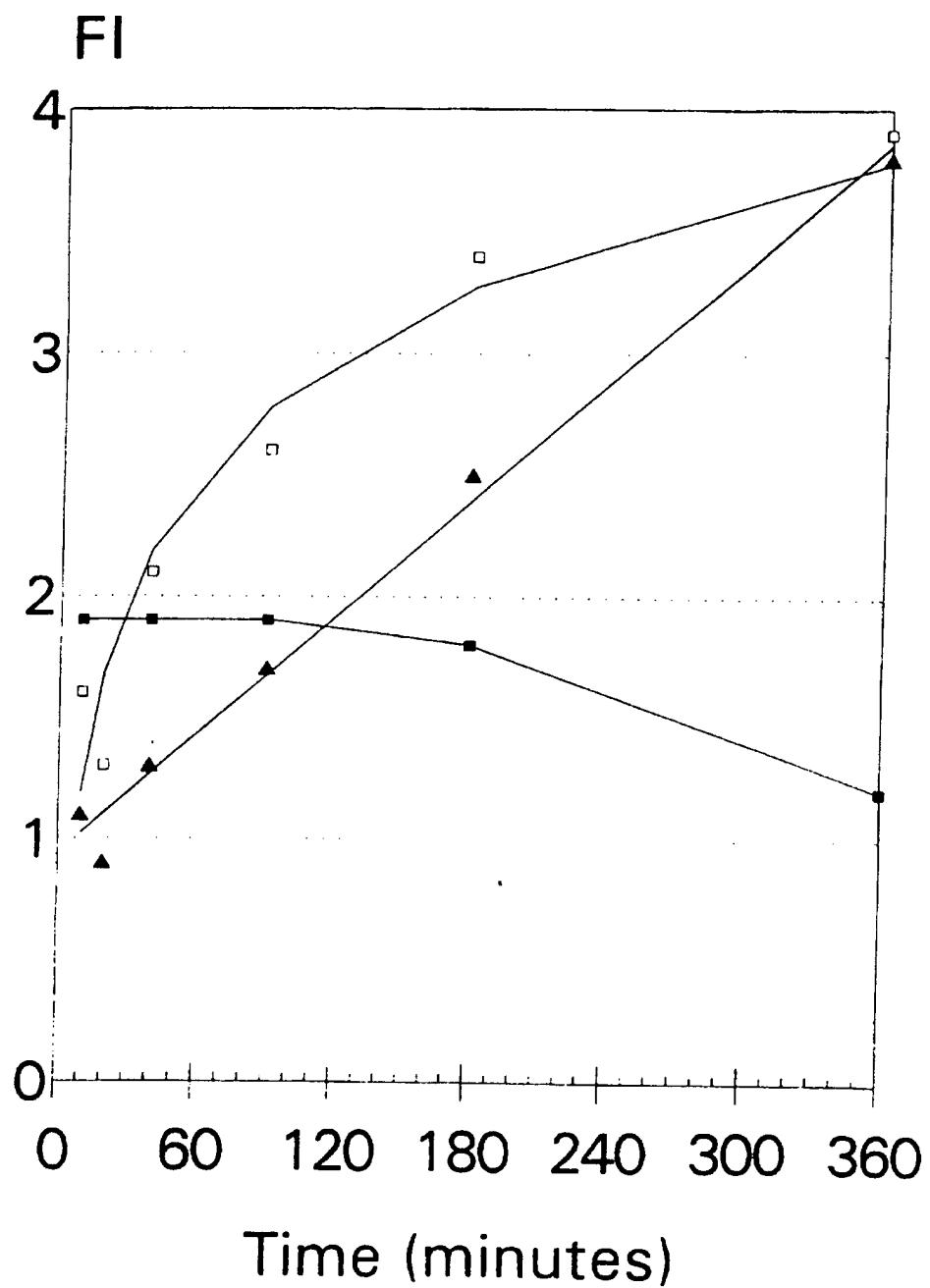
FIG. 3
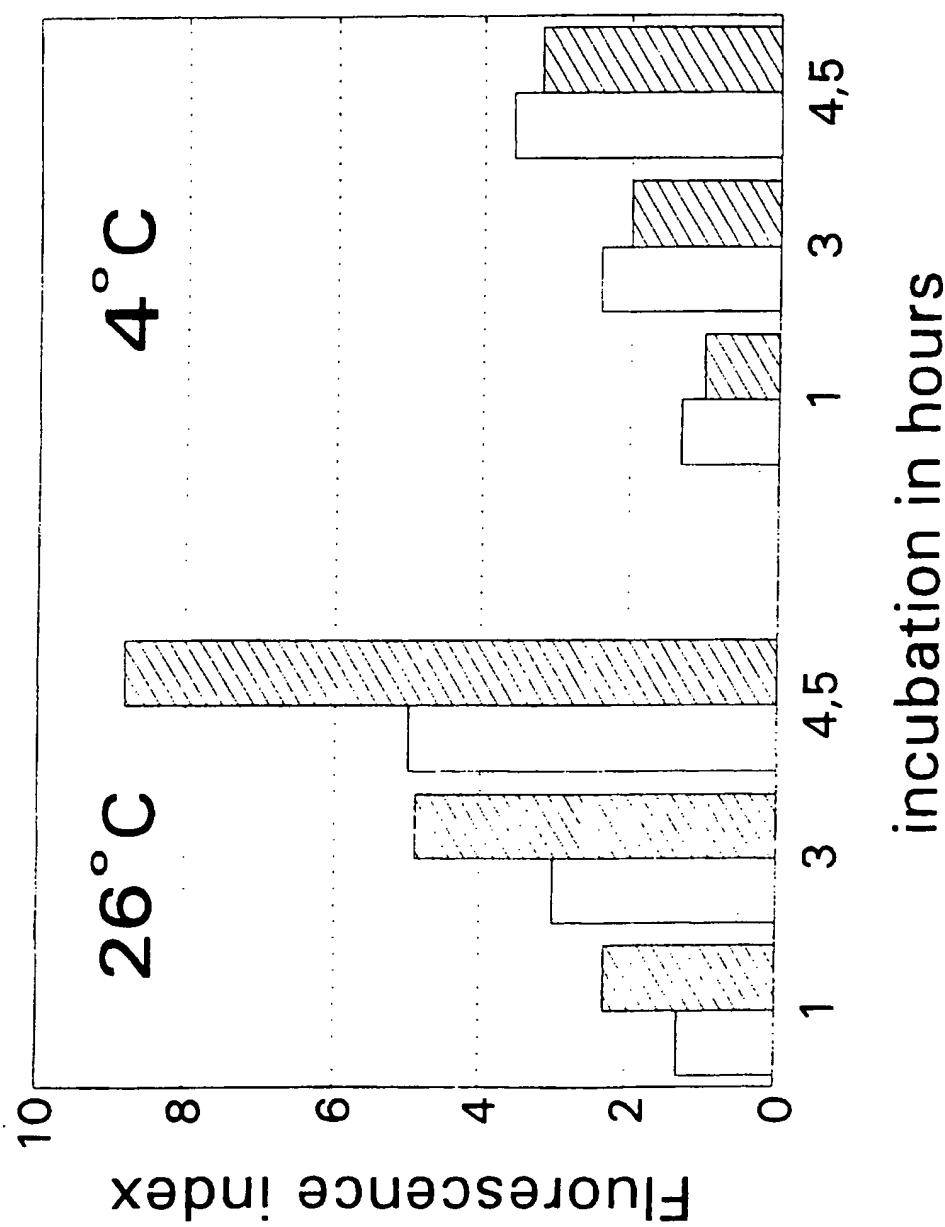
FIG. 4
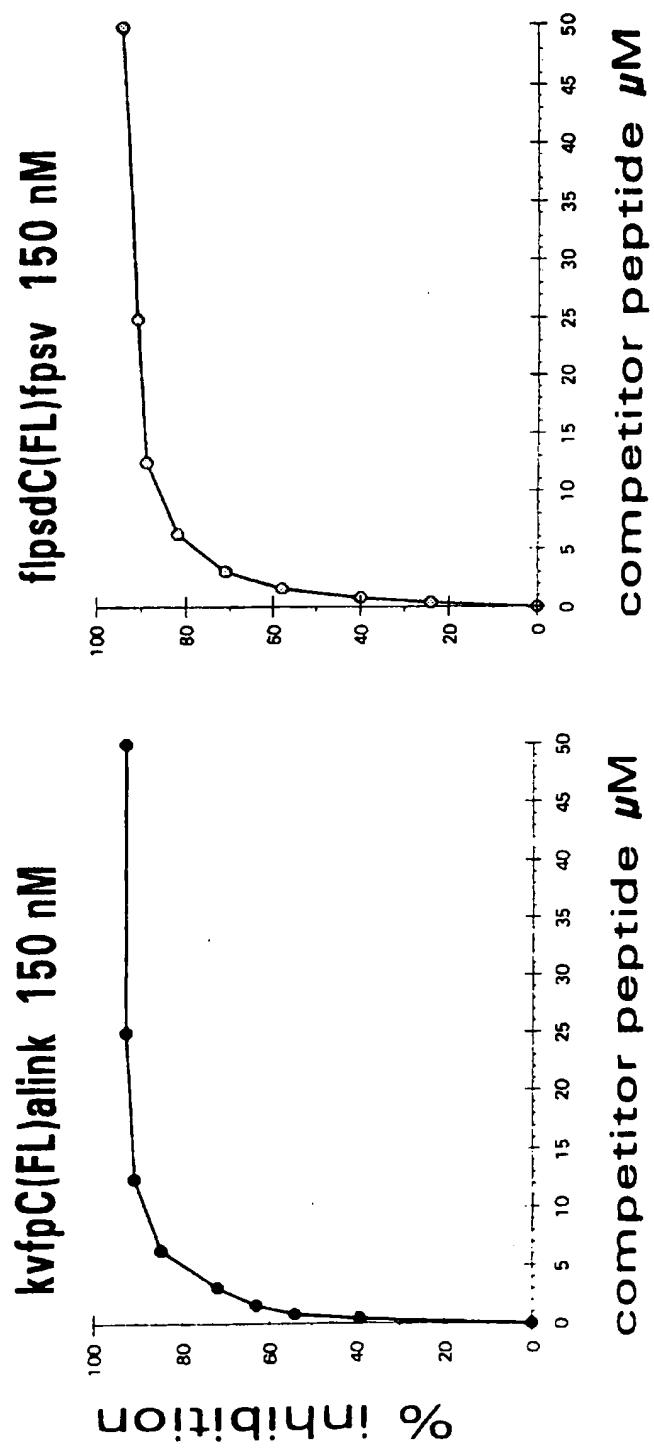
FIG. 5
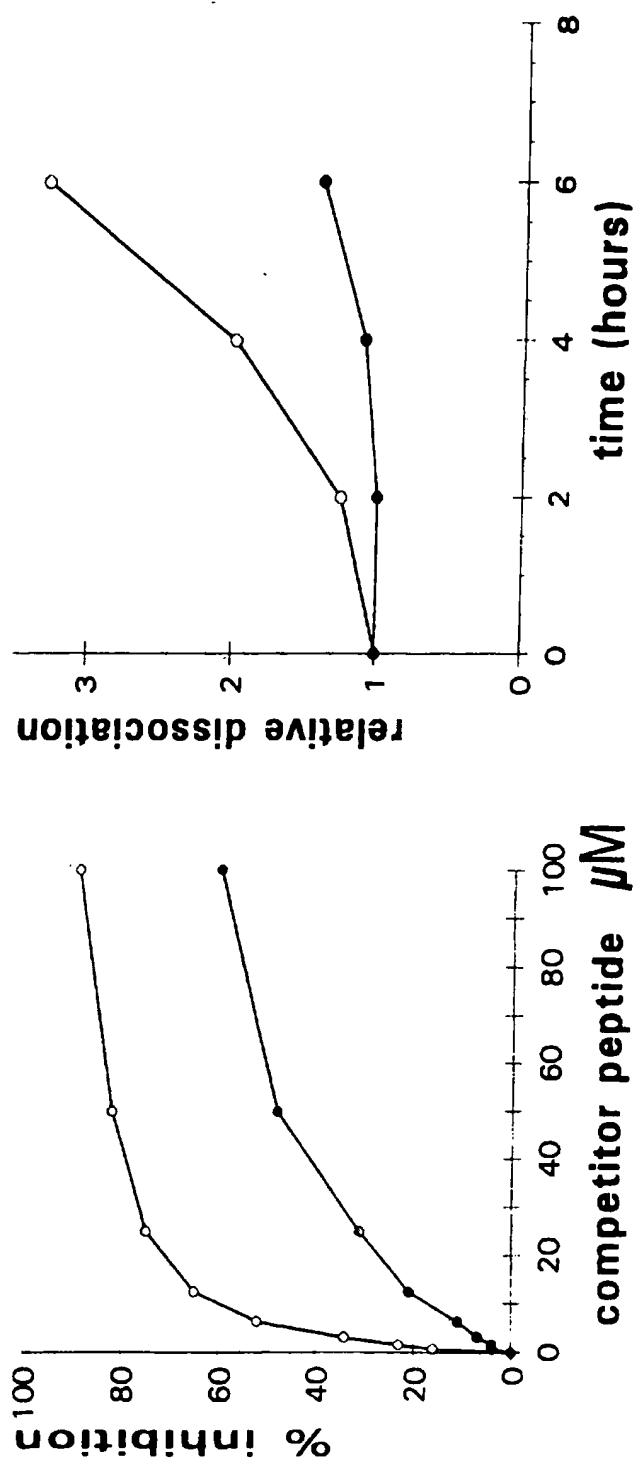
FIG. 6
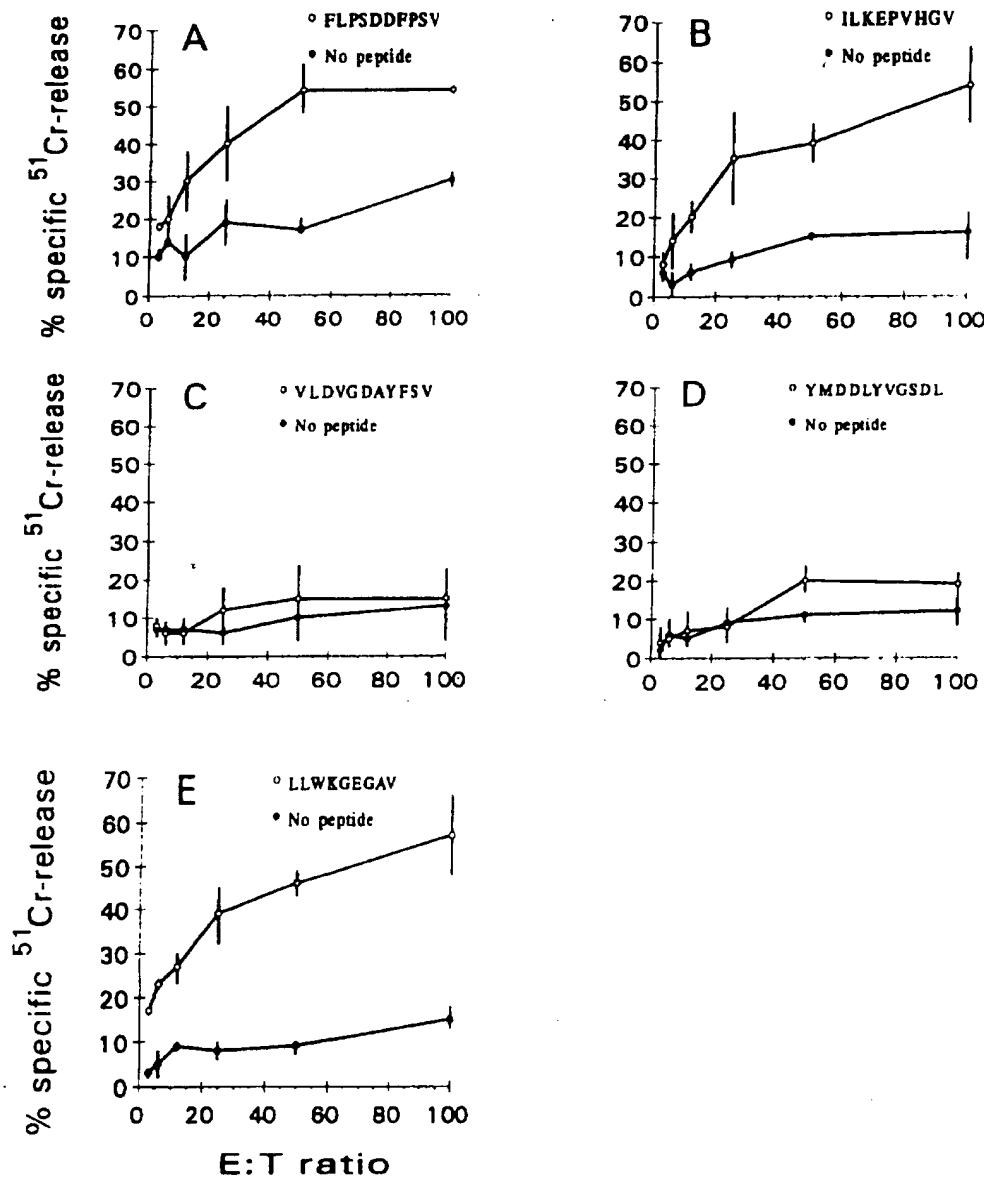
FIG. 7

Recombinant Adenoviruses carrying 'string bead' minigenes

rAd-1

Ad MLP

MNAAASGPSNTPPEIAAARAHYNIVTFAAAIYKKSQHMAAAVNIRNCCYIAAASEQKLISEEDLNN

| Ad5E1A | HPV16 E7 | wt-p53 | Ad5E1B | hu-c-Myc |
| H-2Db | H-2Db | H-2Kb | H-2Db | MAb 9E10 |

rAd-2

Ad MLP

MNAAATLGIVCPIAAAGILGFVFTLAAAYMDLQPETTAAAVNIRNCCYIAAASEQKLISEEDLNN

| HPV16 E7 | Flu-matrix | HPV16 E7 | Ad5E1B | hu-c-Myc |
| HLA-A*0201 | HLA-A*0201 | HLA-A*0201 | H-2Db | MAb 9E10 |
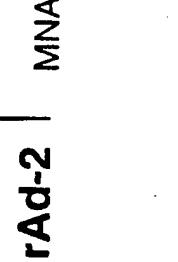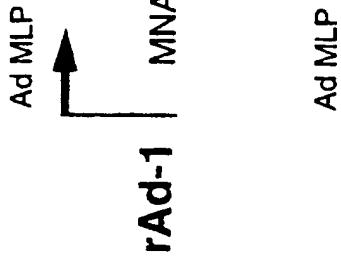
Figure 8

CTL epitopes encoded by string-bead minigene 1 are processed and presented to tumor-specific CTL
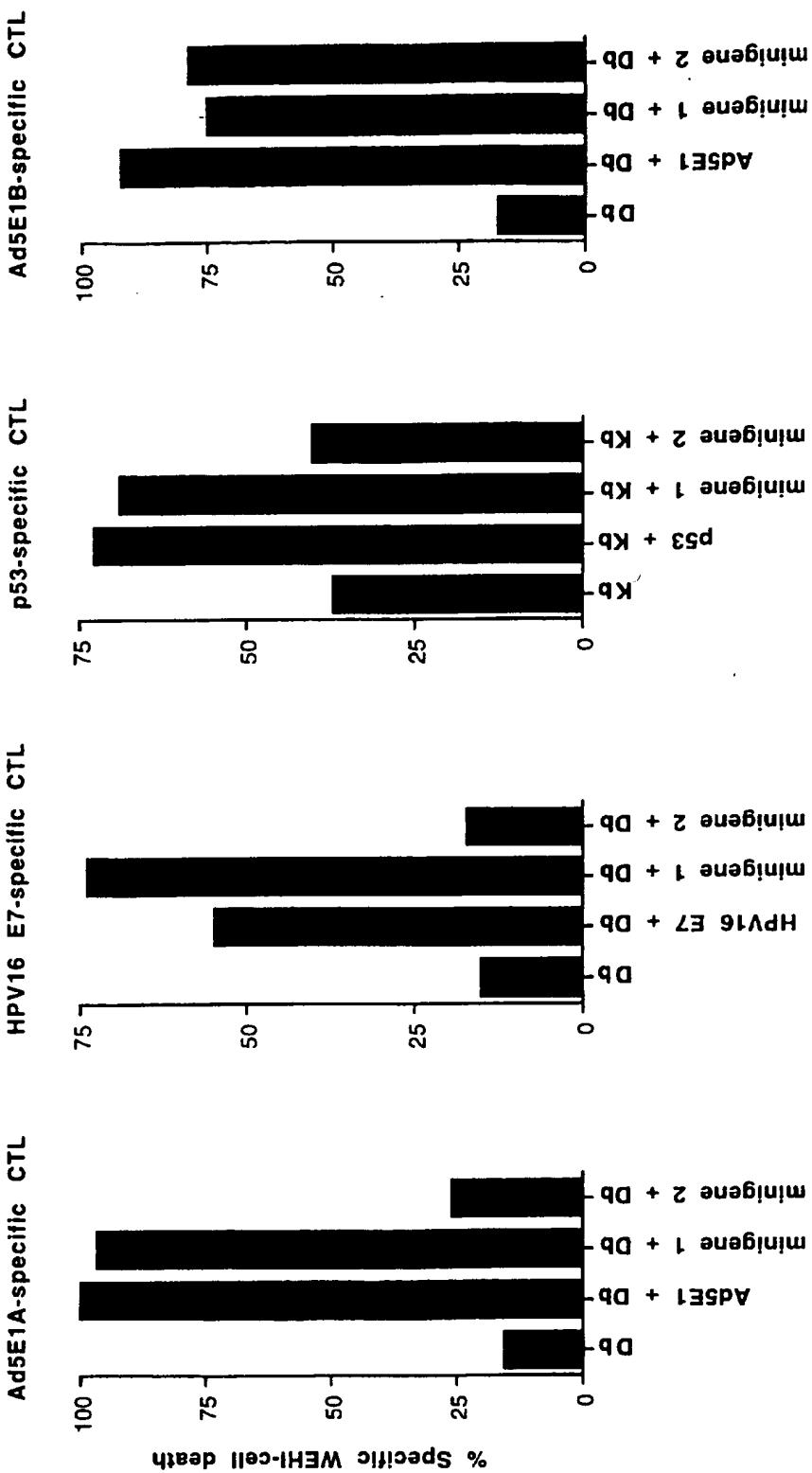
CTL epitopes encoded by string-bead minigene 2
are processed and presented to tumor-specific CTL
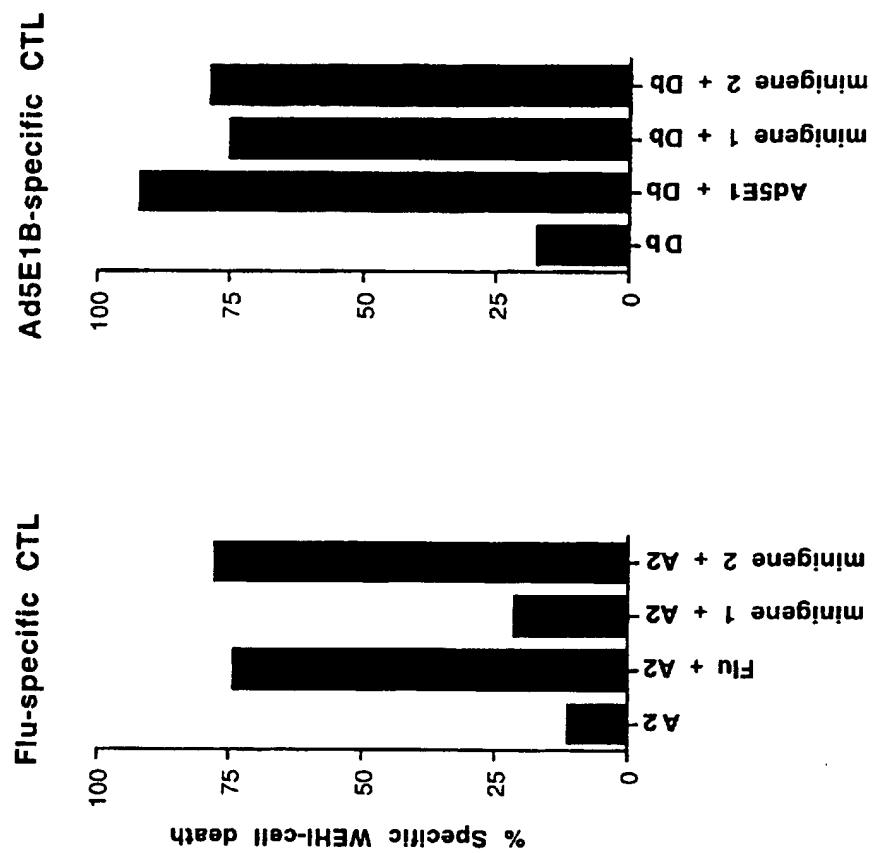
Figure 10

CTL epitopes encoded by rAd in a string-bead fashion
are processed and presented to tumor-specific CTL

Figure 11
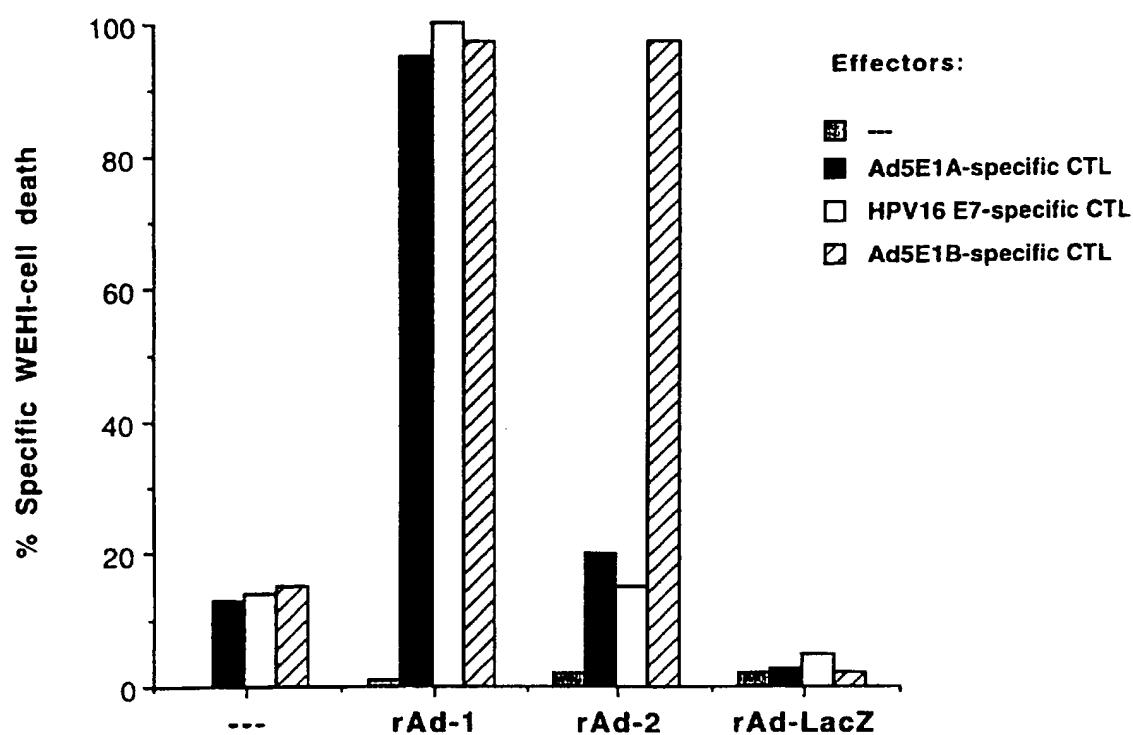
Figure 12

HPV16 E7-specific, H-2^b-restricted CTL immunity induced by vaccination with rAd-1
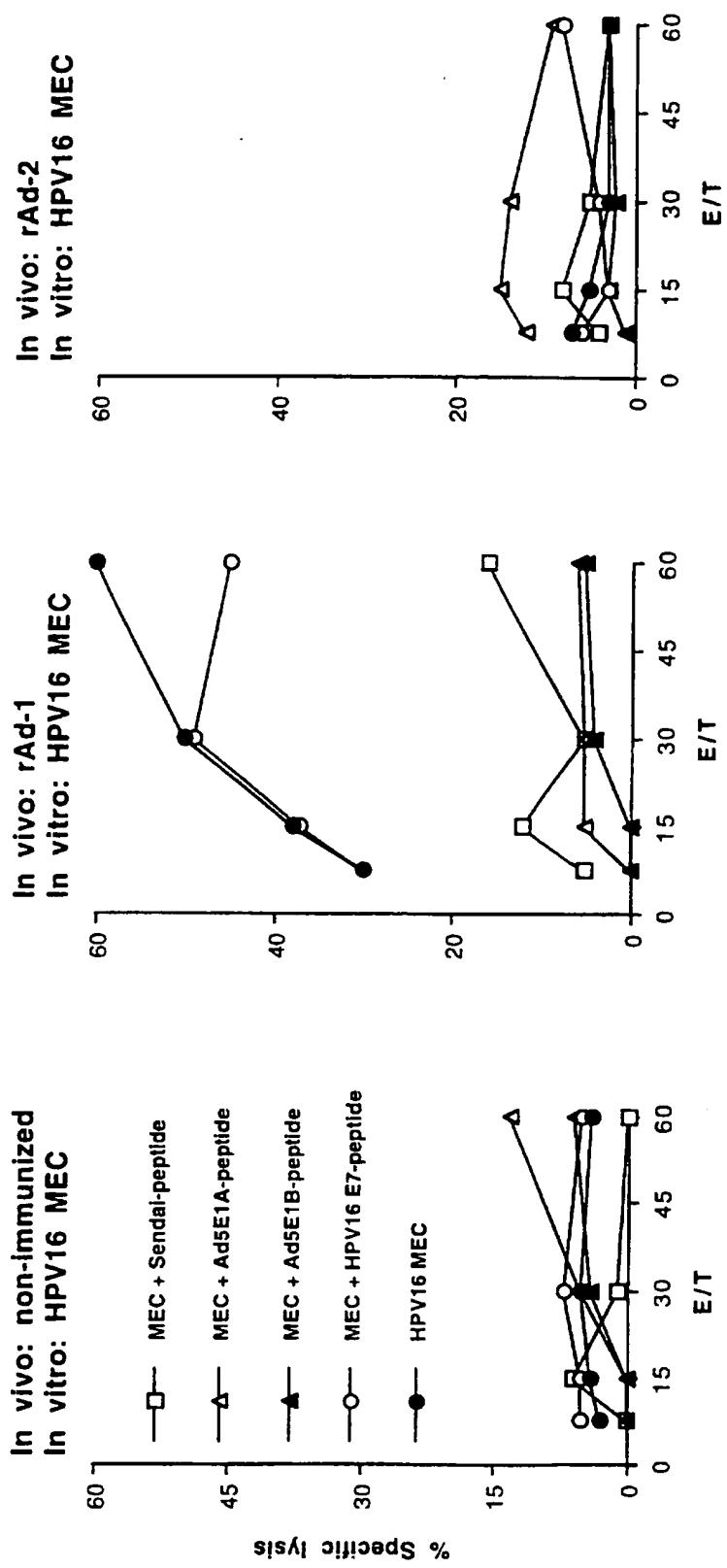
Figure 13

Vaccination with rAd-1 induces protective immunity against Ad5E1A + ras tumors
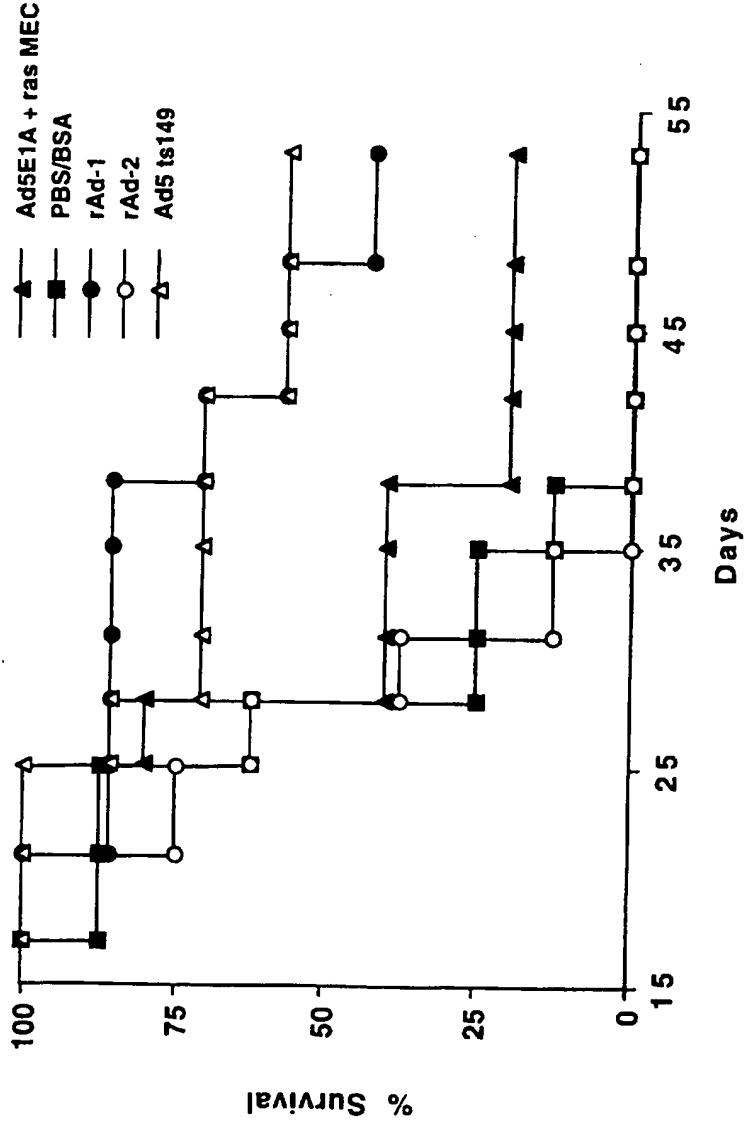
Figure 14

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   G01N33/569   G01N33/557   A61K39/00   C07K14/00   C12N15/00
         C12N15/83

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   G01N   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE (LONDON) (1994), 371(6494), 250-2 CODEN: NATUAS;ISSN: 0028-0836, XP000604820 NELSON, CHRISTOPHER A. ET AL: "Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell" see page 250, left-hand column, line 12 - line 19 see page 250, left-hand column, line 28 - line 32 see page 250, right-hand column, line 1-3 see page 252, left-hand column, line 6 - line 10 see table 2	1-27
X	WO 94 26785 A (US HEALTH) 24 November 1994 see claim 1	20-27

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| [X] | Further documents are listed in the continuation of box C. | [X] | Patent family members are listed in annex. |

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 1997	3 0 -09- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax (+ 31-70) 340-3016	Hoekstra, S

Form PCT/ISA/210 (second sheet) (July 1992)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 156, no. 9, 1 May 1996, pages 1308-1314, XP002040034 VAN DER BURG, S.H., ET AL.: "Immunogenecity of peptides bound to MHC Class I Molecules depends on the MHC-peptide Complex stability" see the whole document ---	1-27
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Y	JOURNAL OF IMMUNOLOGY, vol. 149, no. 11, 1 December 1992, pages 3580-3587, XP000371892 PARKER K C ET AL: "SEQUENCE MOTIFS IMPORTANT FOR PEPTIDE BINDING TO THE HUMAN MHC CLASS I MOLECULE, HLA-A2" see the whole document ---	1-27
Y	WO 94 21287 A (US HEALTH) 29 September 1994 see the whole document ---	13
E	WO 96 22067 A (UNITED BIOMEDICAL INC) 25 July 1996 see the whole document ---	9-27
A	IMMUNOGENETICS (1995), 42(5), 392-7 CODEN: IMNGBK;ISSN: 0093-7711, XP000603776 DAVENPORT, MILES P. ET AL: "An empirical method for the prediction of T-cell epitopes" see the whole document ---	1-27

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ACTA CRYSTALLOGR., SECT. D: BIOL. CRYSTALLOGR. CODEN: ABCRE6;ISSN: 0907-4449, vol. D51, no. 4, 1995, pages 541-549, XP000603826 SCAPOZZA, LEONARDO ET AL: "Molecular dynamics and structure-based drug design for predicting non-natural nonapeptide binding to a class I MHC protein" see the whole document	1-27
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A	JOURNAL OF IMMUNOTHERAPY, vol. 14, no. 2, 1993, pages 121-126, XP002015226 NIJMAN, H.W. ET AL.: "Characterization of Cytotoxic T lymphocyte epitopes of a self-protein, p53 and a non-self-protein, influenzy matrix: relationship between major histocompatibility complex peptide binding affinity and immune responsiveness to peptides." see the whole document	1-27
A	HUMAN IMMUNOLOGY, vol. 43, no. 1, May 1995, pages 13-18, XP000603786 D'AMARO,J. ET AL.: "A computer Program for predicting possible Cytotoxic T lymphocyte epitopes based on HLA Class I Peptide-binding motifs" see the whole document	1-27

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	Internatio Application No PCT/NL 97/00229

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